No. 81

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American Journal of Veterinary RESEARCH

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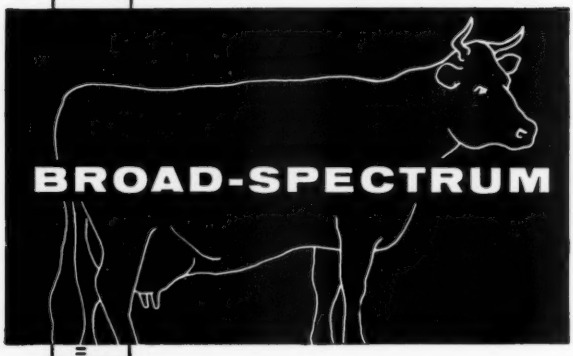

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Volume XXI March, 1960 Number 81

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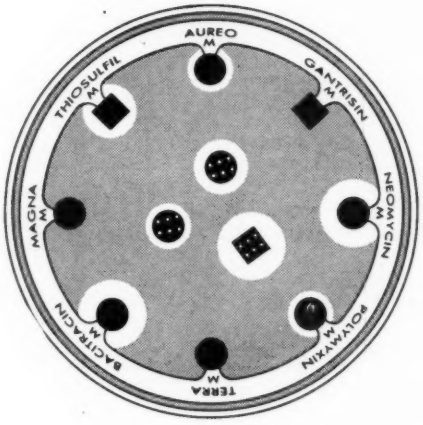

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American Journal of Veterinary Research

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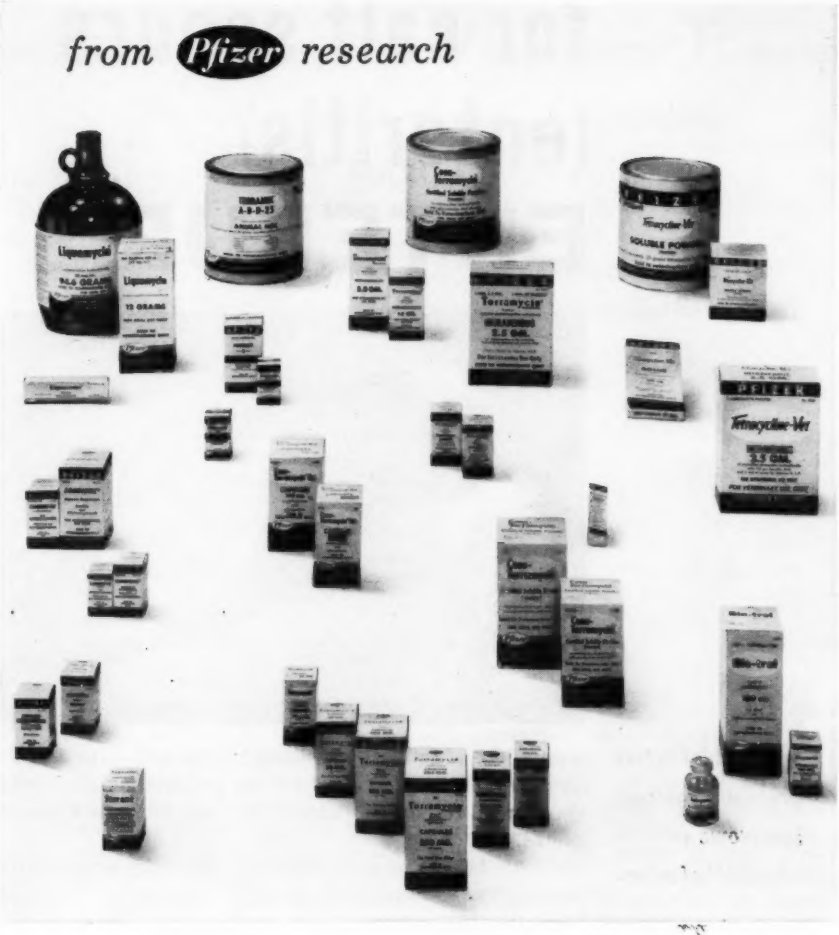

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Bovine Cysticercosis, with Special Reference to the Early Developmental Stages of *Taenia saginata*

ALLEN McINTOSH, Sc.D., and DONALD MILLER, D.V.M.

Beltsville, Maryland, and Phoenix, Arizona

CYSTICERCUS BOVIS infection, or beef measles, has been a continuing problem to cattle feeders in the Southwest for many years.

Unsanitary conditions in a single cattle feedlot may result in an extremely high rate of infection, causing great economic loss to the individual cattle feeder and, if not detected by adequate meat inspection, a health hazard to many thousands of people.

Feeders whose cattle suffer an infection of *C. bovis* can expect to absorb losses of 0.05 to 0.08 cent a pound in marketing those carcasses not condemned outright. Such infection may result when one person infected with the adult tapeworm, *Taenia saginata*, improperly disposes of his body wastes in a manner that permits cattle to have access to the human feces.

Investigations several years ago showed that numerous cases of *C. bovis* infection occurred in the Tucson, Ariz., area when cattle were grazed on a farm pasture which had been irrigated with primary sewage effluent. A high incidence of infection also resulted when cattle were pastured near Phoenix, Ariz., on vegetable fields after the crop had been harvested by Indians. Although satisfactory latrines were available, it was determined that they were not used and that the fields were contaminated by persons later found to be infected with *T. saginata*.

More recently, during 1955, 42.87 per cent of a herd of 1,980 cattle pastured and pen-fed near Phoenix, Ariz. were found to be infected with *C. bovis* when slaughtered. Of the infected cattle, 833 (42.07%) were retained for freezing and 16 (0.80%) were condemned because of heavy infection. A thorough investigation was made to determine the source of the infection. Fecal examinations which were made of all employees at the feedlot and of all available employees who had worked on the farm where the cattle were pastured were negative. However, there had been a considerable turnover of transient employees. In 1956, Miller [2] reported that effluent from a town sewage disposal system of the primary settling type had flowed through pastures in which many of the cattle had been located. However, some of the cattle which became infected had not had access to the effluent but were pastured in other parts of the farm. It was concluded that the use of concrete-lined ditches as watering places for cattle as well as for irrigation provided a source of infection of cattle when the ditches were contaminated by feces from field hands. Examination of specimens of *C. bovis* from the affected animals by personnel of the Beltsville Parasitological Laboratory, Animal Disease and Parasite Research Division, at Beltsville, Md., indicated that infection was occurring after the cattle had been in the feedlot for about two months. Careful investigation

Dr. McIntosh is parasitologist, Helminthological Investigations, Animal Disease and Parasite Research Division, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Md.; and Dr. Miller is veterinarian in charge, Animal Disease Eradication Division, ARS, U.S.D.A., Phoenix, Ariz.

The writers acknowledge the assistance of the Arizona Cattle Feeders Association; Arizona Cattle Growers Association; Dr. Jack A. King, State Veterinarian of Arizona; Dr. Keith T. Maddy, U.S. Public Health Service; Dr. Charles J. Prchal, Meat Inspection Division, ARS; and Arizona State College at Tempe.

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revealed no source of exposure at the feedlot unless certain loads of hay might have been contaminated.

THE PHOENIX FIELD RESEARCH PROJECT

During the course of the investigation of the high incidence of bovine cysticercosis occurring in this herd in 1955, many questions arose in regard to *C. bovis* for which answers could not be found in the available literature. According to Stiles,[5] Zurn [7] and Ostertag [3] observed cysts of *C. bovis* as early as 23 and 18 days after infection, respectively, but their reports were incomplete in that few young larvae were described. Furthermore, no information was available in the literature on the duration of the period between the ingestion of viable eggs by cattle and the first appearance of recognizable cysts in the muscles and on the age at which the cysticerci became infective.

Because of the importance of bovine cysticercosis in Arizona and similarly situated areas, the writers proposed the following project which was to be carried out cooperatively by the Animal Disease Eradication and the Animal Disease and Parasite Research Divisions and the state veterinarian of Arizona. The objectives were: (1) to accurately determine under controlled conditions the characteristics of *C. bovis* of known age; (2) to determine how soon cysticerci may be found after ingestion of viable eggs; (3) to determine the age cysticerci become infectious; (4) to observe the clinical effects of the disease in experimentally infected animals; and (5) to obtain information not available in the literature that would aid in the control of the parasite. Inasmuch as research funds were not available for this work at the time it was proposed, the problem was discussed with representatives of the cattle industry in Arizona. This resulted in the participation of cattle growers and feeders and other agencies in the program.

Results of a study of the early development of *C. bovis*, using material from these experiments, have been reported in abstract (McIntosh [1]). The structure of the younger cysticerci and its relation to the age of the infection are recorded in detail in this paper.

MATERIALS AND METHODS

Thirty-four Hereford, short- to long-yearling steers from the range area of northern Arizona, were placed on feed Dec. 2, 1955, at the Arizona State College farm at Tempe. These cattle were identified by hot-iron brands, I through XXXIV.*

Early in January, 1956, two specimens of *T. saginata* were obtained through the courtesy of the St. Joseph's Hospital staff in Phoenix. The identification was made by members of the staff of the Arizona State Board of Health laboratory.

Gravid segments were placed in gelatin capsules and administered by means of a balling gun to the test animals on Jan. 5, 1956. Animals 17, 18, 23, 24, and 31 to 34, which were not exposed, were used as controls. The remaining odd-numbered animals were given one gravid segment from 1 parasite only, whereas the remaining even-numbered animals were given a segment from each of the specimens. The animals were closely observed to be certain that all capsules were retained.

The 26 exposed cattle were allotted to two equal groups and were put in two similar pens. Two controls were penned with one group and 3 with the other. The remaining 3 controls were put in a third pen, where they were given the same feed as the other cattle. This was done to determine whether Taenia ova might be passed out with the feces of the experimentally infected animals and provide a means of contamination for the controls penned with the exposed cattle.

The experimental animals were frequently observed for any clinical signs of the infection.

In addition to the above-mentioned animals, 3 cattle (20B, 49B, and 4286B) were exposed to infection with *T. saginata* and examined at the Beltsville parasitological laboratory.** The infective material for these experiments was obtained from a hospital in the vicinity of Las Cruces, N. M., and from the Southwestern Medical School, Dallas, Texas.†

Except for the 3 animals infected at Beltsville, the experimental cattle were slaughtered at an abattoir in Phoenix, and the carcasses were processed and inspected like those of any other animal being processed by the establishment. Two controls (33 and 34) were slaughtered Jan. 6, 1956, and the 6 remaining controls were slaughtered in groups of 2 at approximately 12, 24, and 42 weeks. One odd-numbered and 1 even-numbered test animal were then killed for necropsy at weekly intervals through the first month after exposure. Similar pairs were necropsied at 6, 8, 10, 12, 16, 24, 32, 40, 42, and 55 weeks after infection. The Beltsville cattle were slaughtered on postexposure days 11, 36, and 64, respectively (table 1).

Portions of the internal masseter and skeletal muscles, diaphragm, the base of the tongue, heart, lung, liver, and esophagus containing cysticerci were removed and were sent via air mail to the

* Corresponding Arabic numerals are substituted when referring to the animals by number.

** Through the cooperation of Mr. John T. Lucker and Mr. C. H. Hill, parasitologists on the laboratory staff.

† Sent to Beltville by Mr. Rex W. Allen, Animal Disease and Parasite Research Division, State College, N. M., and Dr. Donald V. Moore, Department of Microbiology, Southwestern Medical School, respectively.

Beltsville parasitological laboratory. The specimens from each animal were preserved in 70 per cent alcohol, in 5 per cent formalin, in borax, packed without preservative in an insulated cold pack, and sent without refrigeration, in order to insure the receipt of satisfactory specimens for study.

On arrival at the Beltsville laboratory, the portions of the carcass that were packed in borax were rehydrated in water before the connective tissue cysts and cysticerci were removed for study. The cysts were excised from the surrounding tissue and, except for the older stages, their lengths and widths were recorded. They were opened in a small watch glass containing water or a preservative like that in which the specimen had been immersed. The cysticerci were then measured and examined either directly or after being cleared in lactophenol so that their internal structure could be ascertained by microscopic examination.

The cattle infected at Beltsville were slaughtered at the Laboratory, and the cysts were examined while they were still fresh, after refrigeration, or after preservation in 5 per cent formalin.

RESULTS

The duration of the infection, the dates on which the infected cattle were slaughtered, the results of the experimental infections as ascertained by postmortem examination, the dimensions of the very young connective tissue cysts and their contained cysticerci, and brief descriptions of them and of older cysticerci recovered are recorded (table 1).

No cysticerci were recovered from the 8 controls or from 12 of the 13 odd-numbered animals. This resulted in a considerably larger number of controls than had been anticipated but otherwise did not affect the experiment materially.

In contrast, 12 of 13 even-numbered animals and the animals infected at Beltsville harbored extensive infections with *C. bovis* which, in the majority of instances, involved the skeletal muscles and visceral tissue in addition to the regions of the body that are usually examined to demonstrate the presence of this parasite.

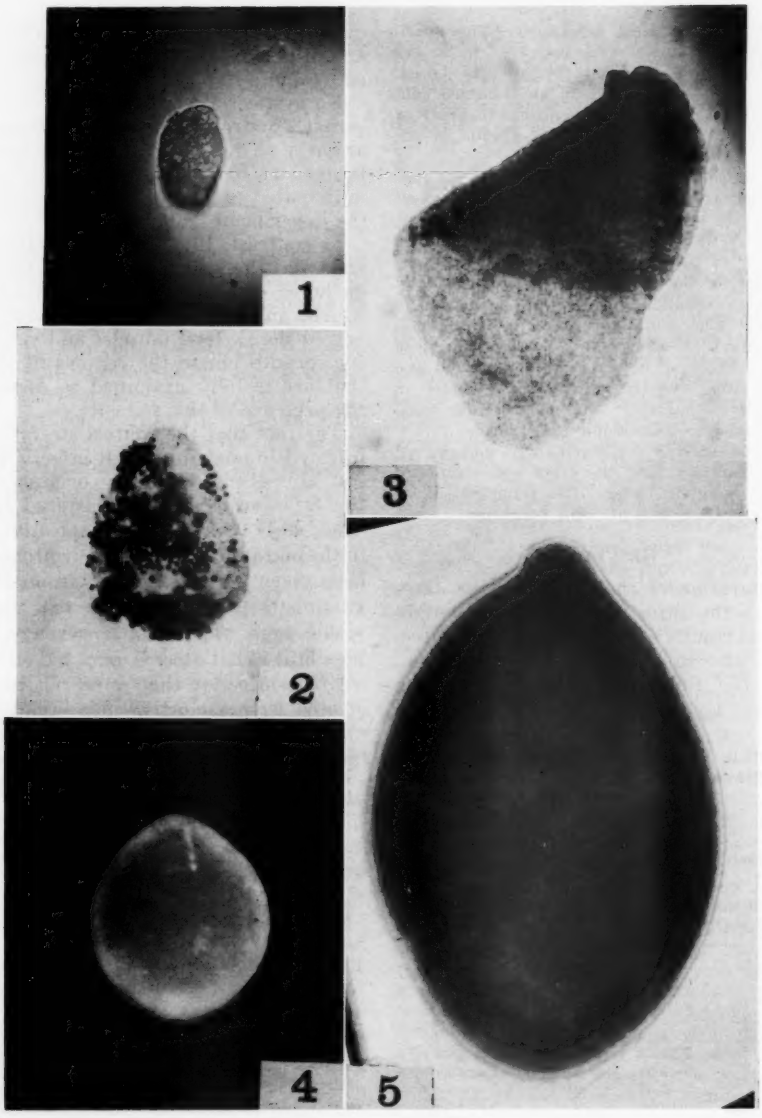
The fact that the control steers were not infected indicated that all infections in the test animals undoubtedly originated from the tapeworm eggs administered to them. Since steer 29 was the only positive animal in the odd-numbered group, it probably had been given the one segment from the worm administered to this group that contained viable eggs. There is, however, a remote possibility that steer 1 may have harbored a latent infection that could not be demonstrated by postmortem inspection.

TABLE 1—Data from Cattle Experimentally Infected with Single Doses of the Eggs of Taenia saginata Showing Correlation Between Morphological Development of Cysticercus bovis and Age of Infection * (Measurements in mm.)

Animal No.	Slaughter date	Age of infection (weeks)	Connective tissue cyst		Cysticercus	Scolex		Diameter of suckers
			Length	Width		Length	Width	
20B	4- 9-56	1½	3	2	0.13–0.15 x 0.09–0.13	....	....	.....
4	1-20-56	2	4	3	0.16–0.17 x 0.1–0.13	....	....	.....
6	1-27-56	3	7	6	1.3 x 0.875	....	....	.....
			9	8	1.65 x 1.38	....	....	.....
			10	7	1.3 x 0.9	....	....	.....
8	2- 3-56	4	6	5	1.15 x 0.82	....	....	.....
			8	4	0.95 x 0.95	....	....	.....
			7	5	1.18 x 1.1	....	....	.....
49B	5- 4-56	5½	8	5	3.0 x 2.0	....	....	.....
10	2-17-56	6	Variable		3.0 x 1.5	0.5	0.4	0.14
12	3- 2-56	8	Variable		5.0 x 2.0	0.7	0.5	0.23
4286B	5- 2-57	9½	Variable		1.5 x 1.23	0.65	0.6	0.28
14	3-16-56	10	Variable		3.0 x 2.25	1.0	0.8	0.3
			Variable		2.62 x 2.55	1.2	0.8	0.3
16	3-30-56	12	Variable		4.55 x 3.7	1.8	1.1	0.3
20	4-27-56	16	Variable		Variable	Variable		0.3
22	6-22-56	24	Variable		Variable	Variable		0.3
26	8-17-56	32	Variable		Variable	Variable		....
28	10-12-56	40	Variable		Variable	Variable		....
29	10-26-56	42	Variable		Variable	Variable		0.3
30	1-25-57	55	Variable		Variable	Variable		....

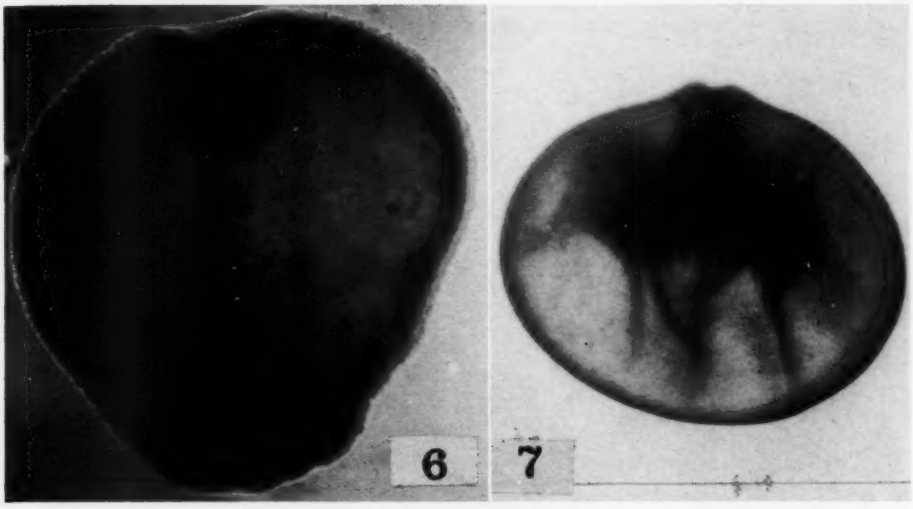
* Only cattle in which cysticerci were found are listed in the table. Control cattle 17, 18, 23, 24, 31, 32, 33, and 34 and exposed cattle 1, 2, 3, 5, 7, 9, 11, 13, 15, 19, 21, 25, and 27 were negative when examined postmortem.

Postmortem findings were: in animal 20B, nodules in diaphragm; in all others, numerous cysts in skeletal muscle and viscera. Many dead cysticerci were found in animals 26, 28, 29, and 30.



Cysticercus bovis

Fig. 1—Age 11 days, 0.15 by 0.09 mm., from diaphragm. x 125.
Fig. 2—Age 2 weeks, 0.17 by 0.13 mm., from masseter muscle. x 192.
Fig. 3—Age 2 weeks, 0.9 by 0.49 mm., a thin folded specimen from lung. x 85.
Fig. 4—Age 3 weeks, 1.65 by 1.38 mm., from diaphragm. x 20.
Fig. 5—Age 3 weeks, 1.3 by 0.875 mm., from heart. x 64.



Cysticercus bovis

Fig. 6—Age 4 weeks, 1.1 by 1.18 mm., from masseter muscle. x 64.
Fig. 7—Age 5 weeks, 3 by 2.5 mm., from diaphragm. x 20.

Steer 2 of the even-numbered group in which cysticerci were not found was killed only one week after experimental infection and undoubtedly harbored tapeworm larvae which either had not reached the muscles or had not been present long enough to elicit the typical connective tissue reaction that would have been visible to the naked eye.

DESCRIPTION OF DEVELOPMENTAL STAGES OF CYSTICERCUS BOVIS

The youngest cysts observed were 11 days old and were about 2.5 mm. in diameter (fig. 1).

From two to 55 weeks after infection in the heavily infected animals, cysts were readily visible in the heart, diaphragm, esophagus, liver, lungs, and in the masseter, gluteal, shoulder, and other skeletal muscles.

At two weeks, the diameter of the connective tissue cysts had increased slightly as compared with those seen on postexposure day 11 and the cysticerci were also slightly larger and were now thin and ovate (fig. 2 and 3).

During the third week after infection, the cysts more than doubled in size and the cysticerci increased from eight to ten times and became saclike (fig. 4 and 5).

Four-week-old cysts were indistinguishable from those observed three weeks after infection, and the size of the cysticerci had not increased appreciably; however, the part of the cysticercus from which the scolex develops had begun to invaginate. There was still no evidence of suckers, the attachment organ of the adult tapeworm (fig. 6).

On postexposure day 36, the connective tissue cysts did not differ markedly from those seen at four weeks, but the size of the cysticerci had more than doubled and the cysticercus contained a spherical process (the developing scolex) which measured about 0.35 mm. in diameter. Suckers had not yet developed (fig. 7).

Six weeks after infection, the connective tissue cysts and the cysticerci within them were of variable sizes, but the scolex of the latter now possessed four cuplike suckers (fig. 8).

Cysticerci 8 and 9 weeks old were oval to transverse elongate and possessed an invaginated scolex with suckers (fig. 9), but the size of the connective tissue cysts (fig. 12) and the cysticerci was still quite variable.

Ten weeks after infection, the scolex had developed a neck portion which was invaginated and the scolex with its developing suckers was invaginated into the receptaculum (fig. 10).

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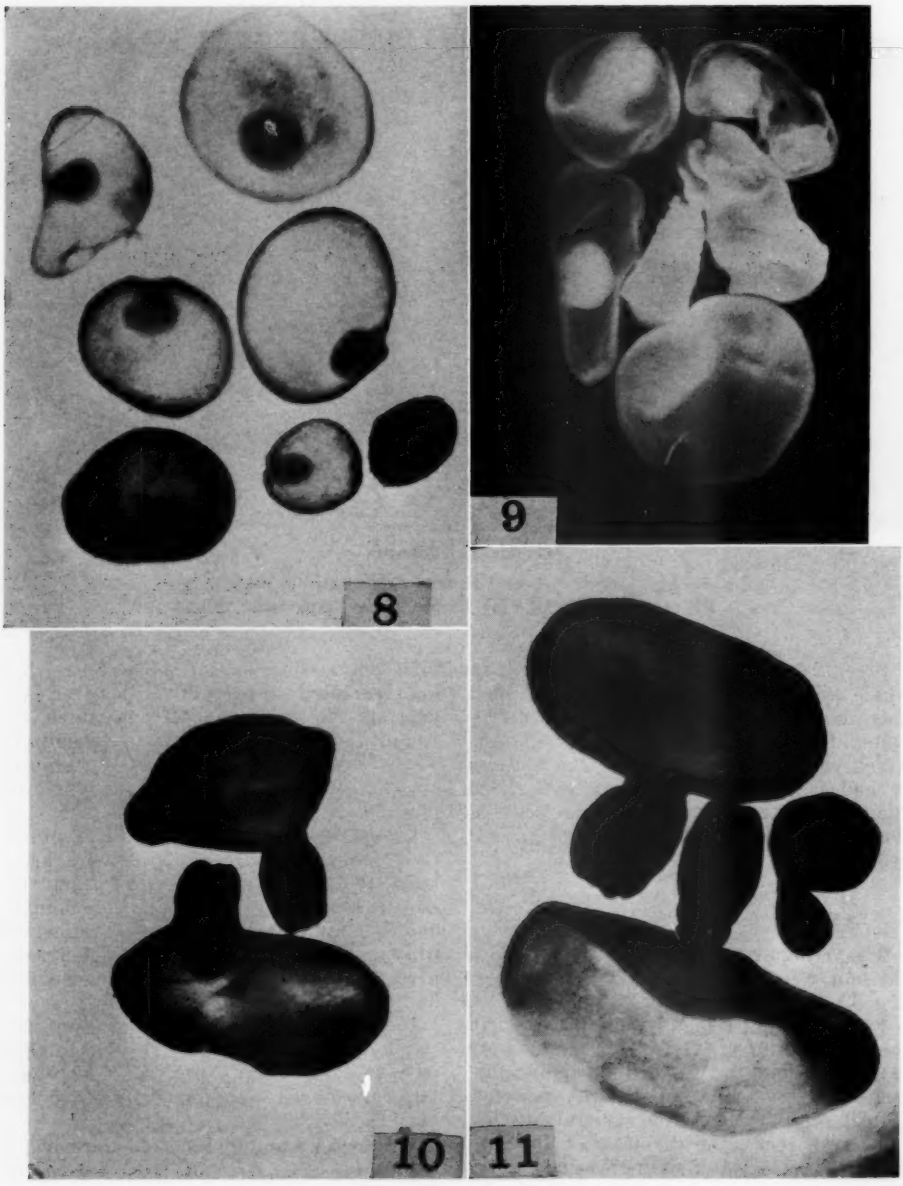
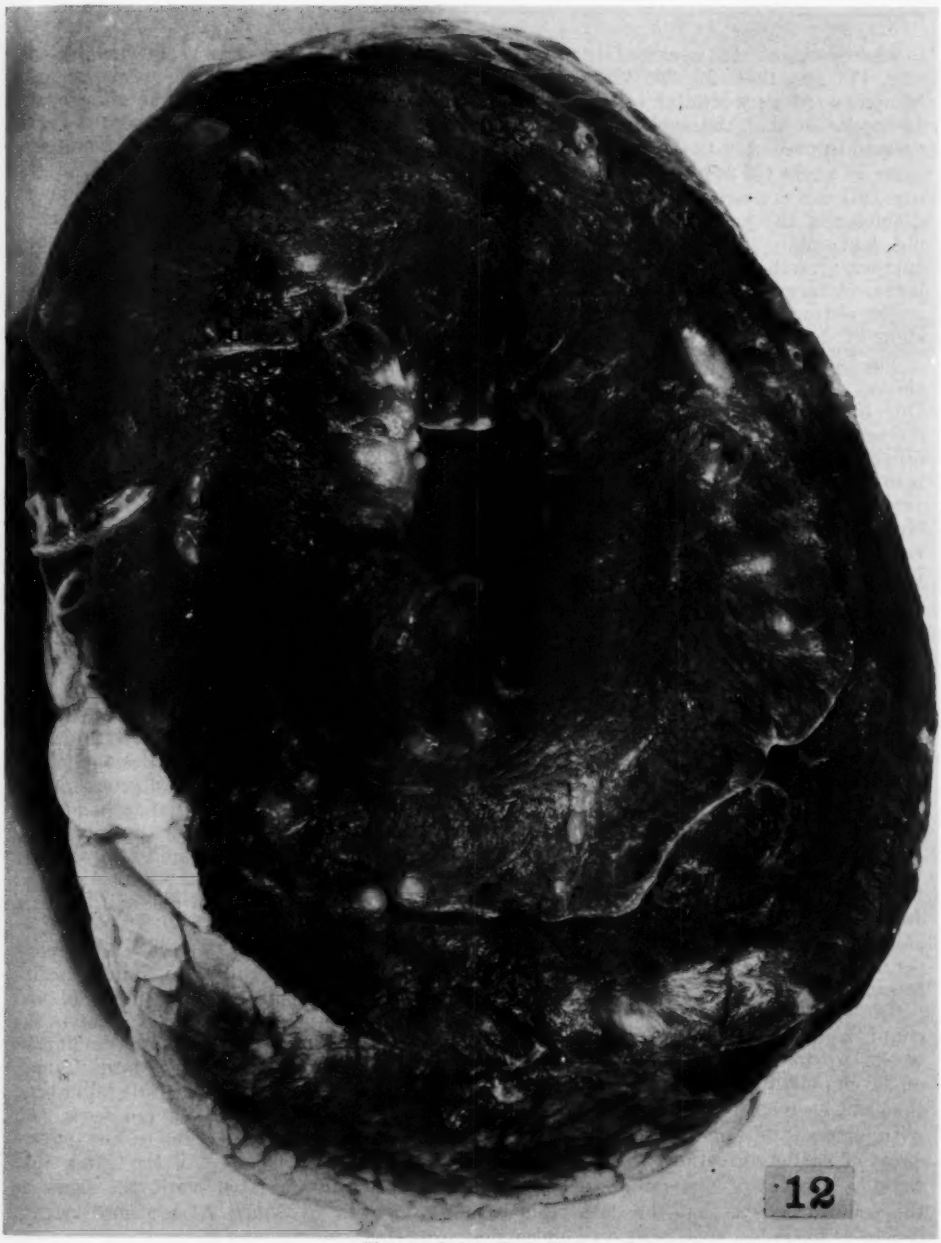


Cysticercus bovis

Fig. 8—Age 6 weeks, size variable, largest = 3 mm. x 10.
Fig. 9—Age 8 weeks, size variable, largest = 3 mm. x 10.
Fig. 10—Age 10 weeks, 3 by 2.25 mm., and 2.55 by 2.62 mm. x 13.
Fig. 11—Age 12 weeks, size variable, largest = 4.55 by 3.7 mm. x 13.



—Photographed by E. C. Hunton, Photographer, Office of Information, ARS

Cysticercus bovis

Fig. 12—Age 64 days, cysts in section of heart. Enlarged slightly.

The cysticerci 12 weeks after infection (fig. 11) and those 16, 24, 32, 40, 42, and 55 weeks old were similar to those seen at 10 weeks in that the suckers had not increased appreciably in diameter. However, those 16 weeks old appeared to be approaching full development as measured by the diameter of the scolex, and those 24 weeks old had many foldlike septa, indicating further growth of the neck portion of the larva. A large number of the cysticerci 32 weeks old or older were dead and showed signs of degeneration.

The cattle which harbored these cysticerci showed few clinical signs of the infection. One had an increased respiration rate on one occasion. At antemortem inspection, another had a rough coat, a drawn up abdomen although it was apparently not off feed, and was thinner than the other cattle. Normal weight increases occurred in the remaining cattle during the period of heavy infection.

DISCUSSION

The foregoing data confirm the findings of Ostertag,[3] as reported by Stiles,[5] that the 18-day-old connective tissue cysts of *C. bovis* measure approximately 4 by 2 mm. and that the scolex and bladder of the contained larvae are not yet differentiated. They also show that the size of the connective tissue cysts and the cysticercus was variable and was of little value in ascertaining the age of the infections, except perhaps to distinguish relatively recent infections from those several weeks old. However, the stage of development of the contained cysticerci appeared to be of some value in ascertaining the ages of the recent infections.

The age of early infections with *C. bovis* could be ascertained within one or two weeks, up to the tenth week, by the stage of development of the cysticercus, in cattle that had been subjected to single infections with eggs of *T. saginata*. Although the effect of earlier infections with *C. bovis* on the development of cysticerci is not known, the writers believe that the data recorded herein will enable meat-packers and cattle feeders to determine fairly accurately the date of a relatively recent infection and thus to trace its source.

No accurate information on the age at which *C. bovis* becomes infective was found in the literature. However, since the neck portion of the scolex was formed by the tenth week and the diameter of the suckers did not increase appreciably in older cysticerci, the writers concluded that the cysticerci were probably infective at about the tenth or twelfth week. The fact that cysticerci 16 weeks old had larger scolices and had undegone some elongation of the neck portion may, however, indicate that they are more mature and, therefore, more infective than younger larvae.

Ostertag,[3] Zurn,[7] and others have reported severe clinical signs and death after the feeding of large numbers of infective eggs of *T. saginata* to cattle. The clinical observations made during the present experiments were largely negative except for the signs shown by the 2 animals mentioned in the preceding section. These signs appeared to be of little value in diagnosing bovine cysticercosis. Normal weight increases occurred in the remaining cattle during the period of heavy infection.

Some believe that cysticerci will be absorbed so that the carcasses will pass meat inspection if infected animals are not slaughtered for several months after exposure to tapeworm eggs. However, after 55 weeks, the carcass of animal 30 showed extensive infection with *C. bovis* and was condemned because of the lesions. This seems to indicate that it is not economically feasible to retain exposed cattle in heavily infested lots with the hope that the cysts will be absorbed. Penfold and Penfold [4] reported that absorption cannot be expected to take place for two and one-half to three years after infection, if at all.

The eradication of bovine cysticercosis requires the cooperation of public health officials and members of the medical and veterinary professions, since it involves the diagnosis of the infection in both the human and animal hosts, treatment of the human host for the removal of the adult tapeworm, the sanitary disposal of human feces, and the prevention of infection of the bovine host. Viljoen's report [6] to the effect that pastures contaminated with the eggs of *T. saginata* in South Africa may remain infectious for as long as two years in the absence of reinfestation, emphasizes the primary importance of preventing the initial infestation of the pasture. The following procedures should be observed if such a program is to be carried out successfully:

1) The feces of all workers on farms or ranches on which cattle are pastured or fattened in feedlots located in areas where bovine cysticercosis is enzootic should be examined for tapeworm eggs, and the infected persons treated for removal of the parasite.

2) Suitable latrines should be available on these farms, and the workers required to use them.

3) Pastures fertilized or irrigated by effluent from sewage systems should not be used for grazing cattle to be slaughtered unless this effluent has been treated to destroy tapeworm eggs.

4) The inspection of all slaughtered cattle should be carried out, preferably under a state or federal system.

Summary

1) The economic importance and epidemiology of bovine cysticercosis in the southwestern United States is reviewed, and observations on the development of *Cysticercus bovis* following experimental infection of cattle are reported.

2) Postmortem examinations were made on 36 cattle, 28 of which were exposed experimentally to infection from one to 55 weeks previously with single doses of infective eggs of *Taenia saginata*.

3) Tapeworm cysts were first observed in the musculature about 11 days after infection.

4) The age of single infections from two to ten weeks old could be correlated with the stage of development of the cysticercus but not with the size of the connective tissue cyst or cysticercus.

5) Cysticerci from 10 to 12 weeks old were believed to be the youngest stages that could be considered on morphological grounds to be capable of infecting the final host.

6) Few, if any, clinical signs developed in the infected cattle that could be considered as resulting from the experimental infections.

7) One steer, slaughtered 55 weeks after infection, was condemned for cysticercosis indicating that it would be economically impractical to keep infected animals long enough to insure the absorption of the cysts and thus to permit the carcass to be passed for human consumption.

8) A program was outlined for the eradication of bovine cysticercosis.

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XUM

The Effect of Bephenium Embonate on Egg Production in *Nematodirus helvetianus* and Other Gastrointestinal Nematodes in Cattle

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AT THE PRESENT time there is no satisfactory treatment for certain genera of gastrointestinal parasites of cattle, including the genus Nematodirus. *Nematodirus helvetianus* has been experimentally shown to be potentially pathogenic in calves,[4] and a severe outbreak with fatalities has been reported from Wyoming.[7] However, the overall pathologic significance of nematodiriasis in cattle is undetermined.[5]

Considerably more information is reported for this disease in lambs and sheep. In these animals it is at times severe and even fatal.[1,2] However, the recent development of bephenium embonate * now presents a possible means of controlling this disease in sheep.[6,8]

This report presents the results of bephenium embonate therapy on calves which were infected with Nematodirus and other gastrointestinal parasites.

MATERIALS AND METHODS

This investigation was conducted using 16 Hereford heifer calves that were born and raised on a ranch near Livermore, Colo. They were maintained in an isolation unit and used as part of a study of respiratory diseases. Within the unit, 4 calves were housed in each room. One calf from each group of 4 was maintained as an unmedicated control. The animals were stanchioned throughout the experiment.

Fecal material for examination was collected either directly from the rectum of an animal or from the concrete floor immediately behind it. Approximately 100 Gm. of feces were collected each time and, after thorough mixing, a 1-Gm. sample was examined. Flotations were made using a saturated solution of sodium chloride. All the nematode eggs recovered were counted and identified as either Nematodirus eggs or as trichostrongylid eggs. Genera of gastrointestinal parasites (other than Nematodirus) represented by trichostrongylid eggs could not be determined on the basis of egg morphology. No attempt was made to recover worms in the fecal material following treatment, since the calves were in strict quarantine; the collection and removal from the premises of large quantities of fecal material for examination was not practical.

Fecal examinations were started 32 days prior to treatment, repeated usually at weekly intervals up to and including the day of treatment, and were also made on the third, tenth, and 22nd days following treatment.

At the time of isolation, two months prior to treatment, the average weight of these 16 calves was 320 lb. (145 kg.). At the time of treatment, the average weight was about 350 lb. (159 kg.). Based on a dosage rate of 250 mg. per kilogram of body weight, each calf was given one treatment consisting of 39.90 Gm. of bephenium embonate, given orally in gelatin capsules.

On both the 31st and 32nd days following treatment, 1 untreated control calf was examined at necropsy in order to determine the variety of genera present in this group of cattle. The abomasum, small intestine, and large intestine were each ligated and removed. These organs and their contents were carefully examined and all the parasites that were found, as well as the unexamined washing from these parts, were preserved in 10 per cent formalin.

RESULTS

The fecal egg counts preceding and following treatment for the 12 medicated calves and the 4 nonmedicated control calves are presented (table 1).

In the 12 treated calves, the Nematodirus eggs per Gm. (e.p.g.) of feces ranged from 2 to 251 on the day of treatment. The trichostrongylid e.p.g. ranged from 1 to 25. Following medication, there were no Nematodirus eggs recovered from 11 of the 12 calves; however, calf 1 had 2 Nematodirus e.p.g. 22 days after treatment. Only 1 or 2 trichostrongylid e.p.g. were detected from 3 of the 12 calves during the post-treatment period; the remaining 9 were negative. At the time of medication, the counts in the 3 control calves having Nematodirus eggs (calves 8, 12, and 16) ranged from 12 to 71 e.p.g. and remained essentially unchanged for the duration of the experiment. The number of trichostrongylid eggs

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This work was supported in part by a grant from Burroughs Wellcome & Co.

* Franten (bephenium embonate), produced by Burroughs Wellcome & Co., Tuckahoe, N.Y.

in the 4 control calves ranged from 2 to 32 on the day the other calves were treated; it also remained about the same.

Both coccidial oocysts and "eggs" of *Moniezia benedeni* were observed in the feces of a number of the 16 calves. The coccidial oocysts were present in small to moderate numbers in 15 of the 16 calves at the time of the first fecal examination 32 days before treatment. However, during the pretreatment period the numbers and incidence of oocysts decreased, and on the day of treatment the feces from only 4 of the 16 calves contained oocysts. The Moniezia eggs appeared inconsistently in the feces of various calves throughout the trial. Both coccidial oocysts and Moniezia eggs were present in the feces of some medicated calves in the post-treatment examinations; their numbers did not seem to have been reduced by treatment.

The necropsy findings in 2 of the control calves are reported (table 2).

None of the treated calves showed clinical signs of toxicosis or sensitivity to bephenium embonate.

TABLE 1—The Effect of Bephenium Embonate on the Fecal Egg Counts of Twelve Treated Calves Compared with Four Untreated Calves

Calf No.	Pretreatment day				Day of treatment	Post-treatment day		
	32	25	18	7		3	10	22
TREATED CALVES (39.9 Gm.)								
1	63/29*	29/25	38/17	26/15	28/13	0/0	0/0	2/0
2	20/8	44/21	37/24	38/14	20/15	0/0	0/0	0/0
3	119/3	75/2	64/6	63/15	74/17	0/0	0/0	0/0
5	100/4	174/13	208/9	160/9	251/25	0/0	0/0	0/0
6	26/1	36/5	64/4	56/6	22/5	0/0	0/0	0/0
7	12/5	15/11	37/13	19/5	17/12	0/0	0/0	0/0
9	3/9	3/10	3/11	4/4	5/14	0/0	0/2	.....**
10	25/0	31/1	61/2	72/3	38/2	0/1	0/0	0/0
11	1/3	2/4	2/6	2/3	2/1	0/0	0/0	0/0
13	89/4	100/2	181/10	79/6	72/1	0/0	0/0	0/0
14	316/16	132/14	222/4	69/10	98/17	0/0	0/0	0/0
15	46/13	28/11	56/8	51/25	41/8	0/0	0/1	0/0
CONTROL CALVES								
4	57/3	191/5	212/5	109/10	0/2	0/3	0/7	0/16
8	80/40	125/26	61/3	75/2	71/32	97/36	98/29	74/43
12	17/24	27/28	36/26	26/5	12/8	11/20	13/6	13/14
16	55/5	51/0	42/2	50/11	52/7	43/15	46/16	79/14

* Numerator = Nematodirus eggs per gram of feces; denominator = trichostrongylid eggs per gram of feces (no generic determination was made).

** Calf 9 died and was not available for examination.

TABLE 2—Nematodes Recovered from Two of the Untreated Calves

Calf No.	Abomasum	Small intestine
Calf 8	*Ostertagia orloffi* (500)*	*Nematodirus helvetianus* (3–4,000)
Calf 12	*Ostertagia orloffi* (200)	*Nematodirus helvetianus* (800)
	Trichostrongylus sp. (1 female)	*Cooperia onchophora* (1–200)

The species of Ostertagia, Cooperia, and Nematodirus were identified by Maybelle B. Chitwood, parasitologist, A.D.P.-A.R.S., Beltsville, Md.

* The numbers in parentheses represent the estimated number of each species of parasite recovered.

DISCUSSION

Because these calves were being used in another research project, the only method that could be utilized to evaluate bephenium embonate was a comparison of pretreatment and post-treatment fecal egg counts. Although this is not the most accurate method for anthelmintic evaluation, the results obtained clearly indicate that bephenium embonate, as administered, had an apparent nematocidal effect upon both the Nematodirus and the other trichostrongylids in these calves. If the action of this chemical were suppressive rather than nematocidal, eggs could have been expected to appear again in the feces by the 22nd day following treatment. However, until such time as critical studies are conducted, one cannot rule out the possibility that the drug permanently sterilized these gastrointestinal parasites without removing them.

In calf 4, one of the untreated controls, sometime between the seventh day prior to treatment and the day of treatment the Nematodirus egg count dropped from 109 to zero and remained at zero during the

remaining period of observation. This spontaneous and complete loss of the Nematodirus infection has not been explained.

Anthelmintic activity of bephenium embonate in sheep against Haemonchus, Trichostrongylus, Cooperia, and Ostertagia has been reported by Copp, *et al.*[3] The nematodes found in 2 untreated calves can be assumed to be essentially the same as those in the treated calves, since all 16 animals in this group were obtained from the same ranch. Therefore, it appears that bephenium embonate is highly effective against *Ostertagia orloffi* and *Cooperia onchophora*, as well as against *N. helvetianus*. These encouraging results indicate that bephenium embonate should be tested against other genera of gastrointestinal nematodes of cattle.

SUMMARY

On the basis of pretreatment and posttreatment fecal egg counts, bephenium embonate given orally at the rate of 250 mg. per kilogram of body weight reduced the Nematodirus eggs per gram (e.p.g.) of feces from a range of 2 to 251 on the day of treatment to zero in 11 of 12 infected Hereford calves. The Nematodirus egg count in the twelfth calf was reduced from 28 on the day of treatment to 2 e.p.g. on post-treatment day 22. The number of trichostrongylid e.p.g. was reduced from a range of 1 to 25 on the day of treatment to zero in 9 of the 12 calves. Of the remaining 3 calves, the most trichostrongylid eggs recovered during the post-treatment examinations was 2 e.p.g.

Based on necropsy findings from 2 of the 4 untreated control calves, the 16 calves were initially infected with *Nematodirus helvetianus*, *Ostertagia orloffi*, *Cooperia onchophora*, and Trichostrongylus sp.

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Studies on Experimental Concurrent Infections of Dairy Calves with Coccida and Nematodes. III. Eimeria spp. and the Threadworm, *Strongyloides papillosus*

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IN A PREVIOUS PAPER,[7] the authors reported that experimental concurrent infections of dairy calves with several species of coccidia (Eimeria) and the small intestinal nematode, *Cooperia punctata*, both of which localize in the small intestine, caused more pronounced clinical signs than did infections with the same species of coccidia or the cooperids alone. The calves used in that investigation were 57 to 62 days old.

In a similar test with coccidia and the medium stomach worm, *Ostertagia ostertagi*, which attack the small intestine and the stomach, respectively, differences between the parasitized groups of calves were not very great.[8] The calves used in that investigation were 43 to 68 days of age.

It has been reported that the intestinal threadworm, *Strongyloides papillosus*, occurs in about 80 per cent of all calves less than four months old in the southeastern states.[10] Therefore, the present experiment was undertaken to compare the results of concurrent infections of younger calves with Eimeria spp. and *S. papillosus*, both of which may commonly be found in the intestines of young calves still on a milk diet. Two tests with calves 13 to 51 days old were conducted during the period of September to December, 1955.

MATERIALS AND METHODS

The methods of maintaining the experimental calves; of procuring, culturing, and administering oocysts; of estimating the numbers of oocysts and threadworm eggs in the feces of the experimental animals; and of determining erythrocyte and leukocyte packed cell volumes were all essentially the same as previously described.[7]

Twelve male grade Jersey calves were utilized. They were obtained from local farms when less than 24 hours old and housed in outdoor, individual, portable pens[3] until the beginning of the experiment. At that time, the calves were allotted to four groups of 3 animals each, so that the average ages of those comprising the various groups were approximately equal. Each group was then housed in an outdoor isolation pen of concrete and hollow-tile construction.[6] They were maintained exclusively on a milk diet until experiment day 25, when they were weaned and fed a commercial milk substitute.

In the first test, the calves of one group (Eimeria group) were each fed 6,000 sporulated oocysts per pound of body weight. Those of a second group, the Strongyloides group, were each exposed percutaneously to infective threadworm larvae. The number of these larvae administered to each calf varied from 200,000 to 400,000, according to the age of the animal. Calves comprising the third group, the Eimeria-Strongyloides group, were each given both parasites, in half the amount administered to single calves in the two preceding groups. The 3 remaining calves were left uninoculated as a control group.

The second test, which utilized the calves surviving from the first test, was begun 49 days later. Each calf was re-exposed to infections with the same kinds of parasites and in the same manner as in test 1.

The same culture of oocysts was used for both tests. The oocysts were given in three nearly equal amounts on experimental days 1, 3, and 4 of each test. The following Eimeria species and percentages of each were used: *E. cylindrica*, 37; *E. ellipsoidalis*, 26; *E. zurnii*, 23 (elliptical form, 15; spherical form, 8); *E. alabamensis*, 8; *E. subspherica*, 4; *E. bovis*, 1; *E. auburnensis*, 1.

Larvae of the threadworm were isolated from charcoal-feces cultures; the feces were collected from an experimentally infected calf. Infections with this nematode can be established in calves much more readily by the percutaneous route than by oral administration of the infective larvae.[10] Consequently, the larvae, in small quantities of liquid, were applied to the skin of the dorsal midline, from which the hair had been clipped. The area was then kept moist for at least 15 minutes to furnish a suitable environment for the larvae to penetrate the skin.

In the first test, the calves of the Strongyloides group were exposed to different numbers of larvae, based on each calf's age as follows: calf 1433, 19 days old, a total of 200,000; calf 1423, 30 days old, 300,000; and calf 1413, 51 days old, 400,000 larvae. The calves given both coccidia and nematodes were exposed to half of these numbers of larvae. In all cases, the larvae were divided into two roughly equal portions per calf and were administered on experimental days 1 and 3.

In the second test, the larvae were administered by a single percutaneous exposure on the first day, with each of the Strongyloides calves being

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exposed to 350,000 larvae, while the Eimeria-Strongyloides calves were each exposed to 150,000.

RESULTS AND DISCUSSION

TEST 1

The comparisons of oocyst counts, days with diarrhea, and growth rates in tests 1 and 2 are shown in tables and the oocyst counts are shown graphically.

In test 1 (table 1), oocyst counts greater than 10,000 per loopful [1] were recorded on an average of more than twice as many days in the Eimeria-Strongyloides group as in the Eimeria group, despite the fact that the calves in the former group were given only about one half the numbers of oocysts given to calves in the other group. The presence of Strongyloides larvae in the Eimeria-Strongyloides group may have enhanced the penetration and development of coccidia that were attacking the intestine in the same region as the larvae.

The high peaks of oocysts discharged during the second week of test 1 (fig. 1) were caused by oocysts of *E. alabamensis*, which has a short prepatent period.[4] The secondary peaks during the third week (fig. 1) were principally due to oocysts of *E. auburnensis* and *E. zurnii*. The greatest average oocyst counts in the *Strongyloides* group and in the control group (a result of natural contamination) were 2,640 and 1,340, respectively.

In test 1, the loss of weight in each group (table 1) during the first week may have been due to the changed environment when the calves were placed in groups of 3 in the concrete-floored pens. The weight gains of all animals were about the same until the fourth week, when calf 1434 (Eimeria-Strongyloides) died on experiment day 31. The youngest calf in each of the other groups also died: 1432 (Eimeria), on day 39; 1433 (Strongyloides), on day 39; and 1435 (control), on day 34. The elimination of these weak calves probably accounted for the improvement in average weights of the survivors during the fifth and sixth weeks (table 1). Hematocrit determinations indicated that no significant changes in erythrocyte or leukocyte numbers occurred in any of the animals.

NECROPSY FINDINGS

Calf 1432 (Eimeria group) was found dead on experimental day 39. It had been weak, had shown typical signs of clinical coccidiosis but without bloody diarrhea, and had gained only 2 lb. during the two weeks prior to death. The poor weight gain and general debility coincided with a high discharge of oocysts of *E. bovis* and profuse diarrhea between days 21 and 28. At necropsy, the gallbladder was distended and discolored. Postmortem changes had occurred in the intestine. No macroscopic

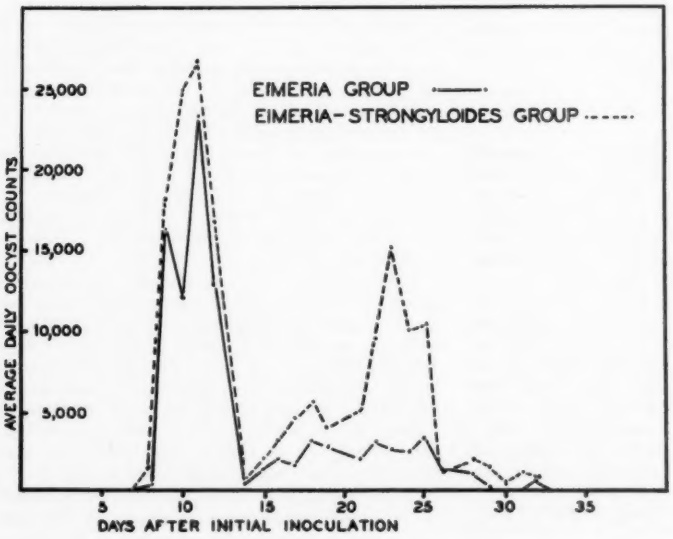

Fig. 1—A comparison of average highest peaks of oocysts per loopful in test 1.

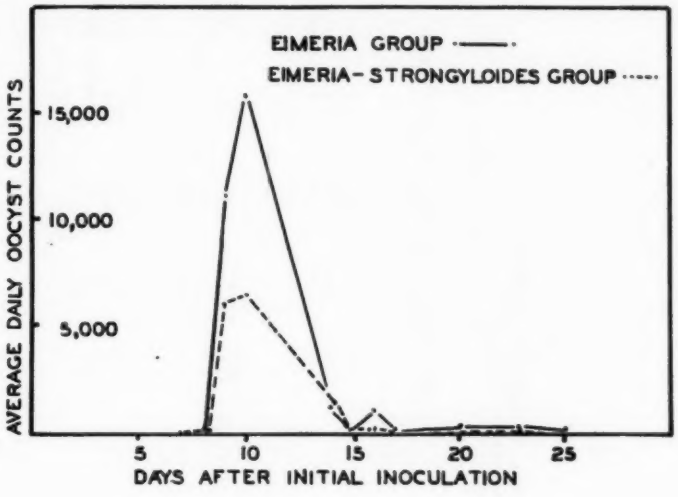

Fig. 2—A comparison of average highest peaks of oocysts per loopful in test 2 (reinfection).

lesions or other signs of coccidiosis were seen in the intestine as the cycles of the coccidia were probably completed before death.

Calf 1433 (Strongyloides group) was also found dead on experiment day 39. On day 10 it was found prostrate, showing extreme respiratory difficulty, and was almost too weak to stand. This is at variance with an earlier report by Vegors,[10] who did not report signs of pulmonary involvement in fatal Strongyloides infections in calves. At necropsy, the lungs showed evidence of recent involvement; however, postmortem changes made it impossible to determine the severity of the pneumonitis. The anterior 15 ft. of the small intestine was hyperemic. A total of 5,640 adult threadworms was recovered from the small intestine. This calf showed a peak fecal egg count of 2,254 eggs per gram of feces (e.p.g.), while the 2 other comparably infected calves had counts of 7,400 e.p.g. each, fifteen days after the initial exposure to threadworm larvae. The fecal egg count for this calf remained high (1,724 e.p.g.) up to the day of death; however, the counts for the other 2 calves infected only with worms dropped rapidly and were 156 and 18 e.p.g., respectively, at the time of the death of calf 1433.

Calf 1434 (Eimeria-Strongyloides group) was killed while moribund 31 days after the initial inoculations. It had been passing large numbers of oocysts of *E. ellipsoidalis* and *E. zurnii* from the second through the third week, followed by great numbers of oocysts of *E. bovis* during the terminal three days. The calf had shown diarrhea on only six nonconsecutive days during the test. At necropsy the lungs were hemorrhagic and were necrotic in some areas. The heart was flabby. The rumen was filled with nearly dry food and had probably lost most of its motility before death. The urinary bladder was distended. Microscopic examination of scrapings from the intestinal walls showed no coccidia in the small intestine and rectum, many merozoites in the cecum and middle and lower colon, and a few in the upper colon. A great many oocysts of *E. zurnii* were found in the upper and lower colon. One oocyst of *E. auburnensis* was seen in the distal end of the cecum.

Only 420 adult worms were recovered from the small intestine. This calf showed a peak fecal egg count of 772 e.p.g. 15 days after exposure to the infective larvae, and the count dropped rapidly to 70 e.p.g. just before the calf was killed. One ensheathed fourth-stage larva was recovered by baermannization[2] of a portion of lung previously minced in a Waring Blendor.

Calf 1435 (control group) was found dead on experimental day 34. The natural infection with coccidia was minimal (table 1), with oocyst counts of over 1,000 per loopful[1] appearing on only four days. Oocysts of *E. cylindrica* and *E. ellipsoidalis* were dominant, with a maximum oocyst

TABLE 1—Infections of Dairy Calves Inoculated with Eimeria spp., Strongyloides papillosus, or both Eimeria and Strongyloides, as Compared with Controls (Test 1)

Calf No.	Age (days)	No. fecal samples examined	No. of days signs of infection were evident in fecal samples						Initial weight	Pounds gained or lost (by weeks) since initial weighing					
			Oocysts*					Diarrhea†		1	2	3	4	5	6
			0–100	100–500	500–1,000	1,000–10,000	10,000+								
EIMERIA GROUP															
1416	45	26	13	1	3	7	2	19	80	2.0	7.0	17.0	25.0	35.0	25.0
1429	24	25	7	2	2	10	4	16	75	1.0	10.0	14.0	19.5	28.0	25.0
1432	19	25	5	4	2	14	0	7	55	−6.0	1.0	5.0	4.0	7.0	(died 39th day)
Av.	29.3	25.3	8.3	2.3	2.3	10.3	2.0	14.0	70.0	−1.0	6.0	12.0	16.2	23.3	.....
STRONGYLOIDES GROUP															
1413	51	26	14	10	2	0	0	0	95	4.0	12.0	21.0	25.0	36.0	32.0
1423	30	26	14	10	2	0	0	0	85	−1.0	11.0	20.0	23.0	30.0	23.0
1433	19	26	9	7	2	8	0	3	55	0.0	2.0	5.0	9.5	9.0	(died 39th day)
Av.	33.3	26.0	12.3	9.0	2.0	2.7	0.0	1.0	78.3	1.0	8.3	15.3	19.2	25.0	.....
EIMERIA-STRONGYLOIDES GROUP															
1414	50	26	10	6	1	5	4	19	105	5.0	14.0	21.0	36.0	48.0	46.0
1422	32	26	4	4	1	12	5	4	70	−5.0	1.0	9.0	22.0	17.0	15.0
1434	14	22	4	1	1	8	8	6	55	−7.0	−6.0	−3.0	4.5	(died 31st day)	
Av.	32.0	24.7	6.0	3.7	1.0	8.3	5.7	9.7	76.7	−2.3	3.0	9.0	17.8	.....	.....
CONTROLS															
1420	35	25	11	7	2	5	0	5	65	3.0	15.0	25.0	27.5	40.0	35.0
1421	34	25	11	10	1	3	0	6	85	−7.0	5.0	12.0	21.5	32.0	32.0
1435	13	23	18	1	0	4	0	7	67	−10.0	−3.0	3.0	1.5	(died 34th day)	
Av.	27.3	24.3	13.3	6.0	1.0	4.0	0.0	6.0	72.3	−4.7	5.7	13.3	16.8	.....	.....

* The numbers represent daily fecal samples showing oocysts per loopful.[1] † Number of times diarrhea was evident (no mucus or blood was seen in this test).

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TABLE 2—Reinfections of Dairy Calves Inoculated with Eimeria spp., Strongyloides papillosus, or both Eimeria and Strongyloides, as Compared with Controls (Test 2)

Calf No.	Age (days)	No. of times signs of infection were evident in fecal samples: Oocysts* 0–100	100–500	500–1,000	1,000–10,000	10,000+	Diarrhea†	Initial weight	Pounds gained (by weeks) since initial weighing: 1	2	3	4	5
EIMERIA GROUP													
1416	94	14	4	0	2	1	5	119.0	9.0	17.0	14.0	13.0	18.0
1429	73	14	2	1	2	2	1	109.0	3.0	8.0	7.0	10.0	13.0
Av.	83.5	14.0	3.0	0.5	2.0	1.5	3.0	114.0	6.0	12.5	10.5	11.5	15.5
STRONGYLOIDES GROUP													
1413	100	20	1	0	0	0	0	140.0	0.0	26.0	7.0	16.0	12.0
1423	79	19	1	0	1	0	2	120.0	0.0	16.0	5.0	16.0	25.0
Av.	89.5	19.5	1.0	0.0	0.5	0.0	1.0	130.0	0.0	21.0	6.0	16.0	18.5
EIMERIA-STRONGYLOIDES GROUP													
1414	99	14	4	0	2	1	7	163.0	1.0	7.0	9.0	16.0	27.0
1422	81	16	1	2	2	0	2	84.0	12.0	13.0	14.0	.....	28.0
Av.	90.0	15.0	2.5	1.0	2.0	0.5	4.5	123.5	6.5	10.0	11.5	.....	27.5
CONTROLS													
1420	84	21	0	0	0	0	0	113.0	3.0	18.0	13.0	18.0	19.0
1421	83	16	5	0	0	0	1	128.0	3.0	17.0	11.0	17.0	22.0
Av.	83.5	18.5	2.5	0.0	0.0	0.0	0.5	120.5	3.0	17.5	12.0	17.5	20.5

Twenty-one samples (daily) were obtained from each calf in 28 days. * The numbers represent daily fecal samples showing oocysts per loopful.[1] † Number of times diarrhea was evident (no mucus or blood was seen in this test).

peak of 3,800 per loopful.[1] Uremia was diagnosed as the probable cause of death.

TEST 2

In test 2 (table 2; fig. 2), the highest peak numbers of oocysts were found in the Eimeria group instead of in the mixed infection group as in test 1 (fig. 1). The dominant species in the response in test 2 was *E. alabamensis*, which, in natural infections, is capable of causing more than one infection in the same calf;[5] calves may be experimentally reinfected as many as four times.[4] It may be that in test 1 the initial, lighter infections of the Eimeria group did not produce as much resistance to reinfection as was developed in the Eimeria-Strongyloides group, and this may account for the reversal of oocyst output in the two groups in the second test. The natural infections in the Strongyloides and in the control groups produced peaks of oocysts per loopful[1] of less than 180 and 140, respectively.

No rise in fecal egg counts occurred in any of the calves in test 2, indicating that the initial infection in test 1 had stimulated a resistance to further infection. Vegors[10] had reported that exposure of calves to *S. papillosus* resulted in a rapid development of resistance to reinfection, and similar observations have been made for this parasite in sheep.[9]

The average gains in weight in test 2 (table 2) were variable. By the end of the fifth week the Eimeria-Stronglyloides calves had gained an average of 27.5 lb., which was about a third more than any other group, including the controls.

As in test 1, there were no significant changes in hematocrit readings following infection.

SUMMARY AND CONCLUSIONS

Two tests were conducted to compare results of concurrent infections with coccidia (Eimeria spp.) and a nematode *(Strongyloides papillosus)* when both attack the same regions of the intestines of young calves still on a milk diet. Three calves in each of three groups were given coccidia, worms, or one-half the numbers of coccidia and worms, while 3 additional calves were left as untreated controls. All calves were kept on a milk diet until experiment day 25 of test 1, when they were fed a commercial milk substitute which was continued until the end of test 2. On experiment day 49, the surviving calves were again inoculated or left as controls, as before (test 2).

In test 1, oocyst counts of higher than 10,000 per loopful[1] appeared on an average of twice as many days in the Eimeria-Strongyloides group as in the Eimeria group, even though calves in the latter group were given twice as many oocysts in the inoculations. Damage produced by the nematodes may have enhanced the penetration and development of the coccidia, since both were in the same general region of the intestine concurrently.

In test 2, the Eimeria group had higher peak numbers of oocysts than the Eimeria-Strongyloides group, the reverse of test 1, possibly because of greater resistance to reinfection developed in the calves with the heavier infections in test 1.

The youngest calf in each group died during the first test.

The average weight gains in all groups were nearly the same by the fourth week in test 1. In test 2, the surviving calves in the Eimeria-Strongyloides group had gained an average of 27.5 lb. by the fifth week, about one third more than any other group.

As shown by the numbers of days when diarrhea was evident, and by the numbers of coccidia produced, the calves were much more susceptible to coccidia in test 1 than in test 2 when they were older and had been exposed to contamination long enough to produce some resistance.

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Studies on Experimental Concurrent Infections of Dairy Calves with Coccidia and Nematodes. IV. Eimeria spp. and the Small Hairworm, *Trichostrongylus colubriformis*

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PREVIOUS EXPERIMENTS with concurrent parasite infections in dairy calves have shown that infection with both coccidia and *Cooperia punctata* is more injurious than a single infection with either of these intestinal parasites.[1] When Eimeria and the medium stomach worm, *Ostertagia ostertagi*, were fed to calves, the development of either parasite and the damage done by it aparently were not enhanced by the presence of the other.[5] When young calves that were still on a milk diet were fed Eimeria or the intestinal threadworm, *Strongyloides papillosus*, or a combination of both at half the single infection rate, there was an indication (except for similar weight gains) that the presence of the worms in the same regions of the small intestine may have enhanced the development of the coccidia.[6]

The following experiments were undertaken to determine the effects on each other, and on the host, of another combination of coccidia and nematodes concurrently attacking the same general area of the small intestine. Eimeria spp. and the small intestinal hairworm, *Trichostrongylus colubriformis*, were utilized.

MATERIALS AND METHODS

The methods of obtaining and administering oocysts and larvae were almost the same as reported before,[4] except for the modifications described. The observations in experiment 1 were made during October and November, 1956; those in experiment 2 in October and November, 1957. In experiment 1, the test calves were 17 to 39 days old (av., 30.6); in experiment 2, from 42 to 58 days old (av., 47.9). After purchase from nearby farms before the animals were 3 days old, these calves were kept in outdoor individual portable pens[2] until three (experiment 1) or five (experiment 2) days before the tests began, after which they were kept in outdoor concrete-floored pens.[3] In experiment 1, the calves that survived 44 days after the beginning of the test were transferred to a pasture naturally contaminated with the parasites being investigated.

In each experiment, 8 calves were allotted to four groups of 2 animals each. Each calf in group 1 was given Eimeria at the rate of 3,000 oocysts per pound of body weight; in group 2, Eimeria at the same rate plus 70,000 larvae of *T. colubriformis;* and in group 3, half the numbers of coccidia and larvae given in group 2. Calves in group 4 were maintained as untreated controls. Groups 2 and 3 were included for comparison between additive and synergistic activity of the two classes of parasites.

Eimeria Infection.—In both experiments, the cultures of Eimeria oocysts were obtained from infected calves at this laboratory. In the first experiment the culture was predominantly *Eimeria bovis*, which made up approximately 85 per cent of the total, with *E. auburnensis, E. ellipsoidalis, E. alabamensis, E. zurnii, E. canadensis,* and *E. cylindrica* constituting the remainder. The oocysts were given orally in three roughly divided portions on October 1, 2, and 4, 1956, in the manner described previously.[4]

In experiment 2, the Eimeria culture contained the following percentages of the species listed: *E. ellipsoidalis,* 38; *E. bovis,* 27; *E. zurnii,* 10; *E. cylindrica,* 9; *E. canadensis,* 8; *E. auburnensis,* 7; and *E. alabamensis,* 1, with *E. bukidnonensis* presence as a trace. The suspension of oocysts was so concentrated that only 5.6 to 14.0 ml. was given to each calf. A plastic pipette was devised from polyethylene tubing that was drawn out after heating to make a pipette about 100 mm. long, with both ends tapering to about 4 mm. in diameter, allowing a 15-ml. rubber bulb to be attached. This was used to place the suspension on the back of the calf's tongue. The suspensions were given on Oct. 16, 17, and 18, 1957.

Trichostrongylus Infection.—*Trichostrongylus colubriformis* was isolated from naturally infected cattle, and then maintained in pure culture by continuous passage through calves raised free of helminths.[2] The numbers of infective larvae used in the inoculations were determined by dilution counts. These larvae were administered in water to the calves *per os* by means of a metal blowpipe attached to a 15-ml. rubber bulb. In both experiments, each calf given the full dose of larvae (group 2) was inoculated with 70,000 (± 500) larvae, while those in the half-dose groups were given one-half the above number. In both experi-

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ments, larvae were given in one dose on the initial day.

RESULTS AND DISCUSSION

Experiment 1.—In experiment 1 (table 1) calf 1503 of group 2 died 29 days after infection. Up to that time, the calves in group 3 (half-dose) had diarrhea 68 per cent of the times sampled, as compared with 47 per cent for group 1, 32 per cent for group 2, and 8 per cent for group 4 (controls). In the final two weeks of the experiment, the presence of diarrhea was essentially the same for all groups, with the exception of the survivor from group 2. This calf had diarrhea on only one of the remaining 14 days.

The calves given only half the numbers of oocysts (group 3) had high counts of oocysts on more days than those given the full dose (table 1). The former group showed oocyst counts of over 1,000 per loopful [1] in 22 (28.5%) of the 77 daily samples examined, while the latter group showed this high count in only 14 (23.3%) of the 60 daily samples.

Except for the second week, the controls made consistently better weight gains than the other calves (table 1). By the third week, the Eimeria-treated calves had made smaller average gains than the other infected animals. Group 2 calves were omitted from the weight comparisons after the third week because of the death of calf 1503. During the fifth and sixth weeks, the average weight gain (table 1) of the group 1 calves was about equal to or slightly better than that of the group 3 calves, indicating that the peak effects of the coccidia had dissipated but that the worms continued to exert a retarding effect on the rate of gain.

As stated previously, on experiment day 44 the surviving calves were placed together on a naturally contaminated pasture. At that time, both group 3 calves were in extremely poor condition as compared with the others. Both had rough coats and showed general debility. They continued in poor condition and died—calf 1499 on day 49, five days after being placed on pasture, and calf 1500 on day 56.

Calf 1496 (group 1) developed diarrhea on experiment day 18 and continued to have diarrhea until day 34, with only three days during this interval when the feces were not fluid. The diarrhea was associated with high oocyst counts of *E. bukidnonensis*, followed by high counts of *E. bovis* oocysts.

Calf 1508 (group 1) had high counts of oocysts of *E. ellipsoidalis* on experiment day 16, with very high counts of *E. bovis* on days 21 and 22, when diarrhea began. *Eimeria zurnii* oocysts became dominant on day 25 and remained dominant until day 33. Mucus and blood were evident during the early discharges of this species, which is one of the most destructive in cattle.[7] Weight gains (table 1) were poor or negative during the peaks of oocyst discharge during the third and fourth weeks.

Calf 1498 (group 2) had low oocyst counts as compared with the calves of group 1. A count of over 1,000 oocysts of *E. bovis* per loopful occurred only on experiment day 16. *Eimeria canadensis* and *E. auburnensis* were also present, but in comparatively small numbers. This low response to the inoculation was associated with fairly good weight gains by this calf.

Calf 1503 (group 2) had high counts of oocysts of *E. ellipsoidalis* beginning on experiment day 17, followed by high counts of *E. canadensis* and then *E. bovis*. It had severe diarrhea during the last nine days of life, and blood and mucus appeared. There was a weight loss of 6 lb. during the week prior to its death on day 29 (table 1). For two days before death some oocysts of *E. bovis* were discharged in clusters surrounded by tissue, resembling the way some are found in sections of intestinal glands.[7] At necropsy 7,960 *T. colubriformis* were recovered from the small intestine. The gallbladder was abnormally distended, as previously described [8] in fatal trichostrongylosis of calves.

Calf 1499 (group 3) began to show diarrhea on experiment day 17, and it continued until day 40. High peaks of oocysts commenced on day 24 with *E. canadensis*, followed in greater numbers by those of *E. auburnensis* and *E. ellipsoidalis*. There was almost no gain in weight between the third and sixth weeks (table 1), the period during which the greatest numbers of oocysts were discharged. This calf died on day 49, five days after being placed on pasture. There were numerous crateriform lesions in the abomasum, and the upper duodenum was covered with a catarrhal membrane. Approximately 15,120 *T. colubriformis* oocysts were present in the small intestine, as shown by examination of aliquot parts.

Calf 1500 (group 3) started showing diarrhea on experiment day 12 and it con-

TABLE 1—A Comparison of Calves Exposed to Eimeria spp. or Combinations of Eimeria and Trichostrongylus colubriformis and Controls (Experiment 1)

Calf No.	Age (days)	Av. No. daily fecal samples examined	No. of times signs of infection were evident in fecal samples (av.)								Initial weight	Pounds gained or lost (by weeks) since initial weighing					
						Oocysts*											
			Mucus	Blood	Diarrhea	0–100	100–500	500–1,000	1,000–10,000	10,000+		1	2	3	4	5	6
GROUP 1 (EIMERIA)																	
1496	39	39	0	0	17	14	5	5	15	0	55	8	13	15	16	18	27
1508	17	38	3	2	19	8	6	7	11	6	55	10	19	20	17	31	41
Av.	28.0	38.5	1.5	1.0	18.0	11.0	5.5	6.0	13.0	3.0	55.0	9.0	16.0	17.5	16.5	24.5	34.0
GROUP 2 (EIMERIA-T. COLUBRIFORMIS)																	
1498	35	39	1	0	10	21	11	3	4	0	65	13	18	28	36	42	53
1503	28	21	2	6	9	5	4	2	9	1	63	12	25	19	Died on 29th day		
Av.	31.5	30.0	1.5	3.0	9.5	13.0	7.5	2.5	6.5	0.5	64.0	12.5	21.5	23.5	....	....	....
GROUP 3 (EIMERIA-T. COLUBRIFORMIS, HALF-DOSE RATE)																	
1499	35	38	0	0	26	23	7	3	5	0	57	10	21	27	27	29	28
1500	33	39	0	0	26	17	3	2	16	1	60	2	27	18	11	19	29
Av.	34.0	38.5	0.0	0.0	26.0	20.0	5.0	2.5	10.5	0.5	58.5	6.0	24.0	22.5	19.0	24.0	28.5
GROUP 4 (CONTROLS)																	
1506	25	39	0	0	1	23	6	3	7	0	57	8	15	24	23	29	38
1501	33	38	1	0	5	15	7	4	12	0	65	19	26	33	46	47	62
Av.	29.0	38.5	0.5	0.0	3.0	19.0	6.5	3.5	9.5	0.0	61.0	13.5	20.5	28.5	34.5	38.0	50.0

* Oocysts per loopful.[1]

XUM

TABLE 2—A Comparison of Calves Exposed to Eimeria spp. or Combinations of Eimeria and Trichostrongylus colubriformis and Controls (Experiment 2)

Calf No.	Age (days)	Av. No. daily fecal samples examined	No. of times signs of infection were evident in fecal samples (av.)								Initial weight†	Pounds gained or lost (by weeks) since initial weighing					
						Oocysts*											
			Mucus	Blood	Diarrhea	0–100	100–500	500–1,000	1,000–10,000	10,000+		1	2†	3†	4	5	6†
GROUP 1 (EIMERIA)																	
1537	58	34	0	0	23	21	8	2	3	0	88	2	8	8	18	22	36
1550	42	35	0	0	24	11	11	3	10	0	70	4	10	10	16	36	37
Av.	50.0	34.5	0.0	0.0	23.5	16.0	9.5	2.5	6.5	0.0	79.0	3.0	9.0	9.0	17.0	29.0	36.5
GROUP 2 (EIMERIA-T. COLUBRIFORMIS)																	
1539	55	17	2	1	13	8	3	3	3	0	82	8	13	−5	Died on 21st day		
1549	42	18	1	1	10	1	4	0	12	1	72	10	9	−1	Died on 20th day		
Av.	48.5	17.5	1.5	1.0	11.5	4.5	3.5	1.5	7.5	0.5	77.0	9.0	11.0	−3.0	.....	.....	.....
GROUP 3 (EIMERIA-T. COLUBRIFORMIS, HALF-DOSE RATE)																	
1540	51	36	2	0	12	21	8	4	3	0	83	13	19	15	35	37	45
1547	43	35	0	3	21	11	4	5	15	0	71	9	9	9	1	9	21
Av.	47.0	35.5	1.0	1.5	16.5	16.0	6.0	4.5	9.0	0.0	77.0	11.0	14.0	12.0	18.0	23.0	33.0
GROUP 4 (CONTROLS)																	
1542	50	35	1	1	1	24	8	2	1	0	81	−1	14	17	25	29	42
1551	42	36	0	0	9	20	8	2	6	0	81	15	13	15	34	35	43
Av.	46.0	35.5	0.5	0.5	5.0	22.0	8.0	2.0	3.5	0.0	81.0	7.0	13.5	16.0	29.5	32.0	42.5

* Oocysts per loopful.[1] † Average weights made on two consecutive days; the others are single weights.

tinued until day 32. The initial abnormal discharge was correlated with the appearance of the first discharge of oocysts of *E. alabamensis*, which continued for five days, followed by high counts of *E. ellipsoidalis* and *E. bovis* oocysts for five and seven days, respectively. The greatest fecal fluidity corresponded with the appearance of *E. bovis*. Beginning at two weeks a weight loss was associated with the peak appearances of oocysts, and the loss was not recovered until the sixth week (table 1), which was after the coccidial infections had terminated. This calf died on day 56, 12 days after going on pasture. Examination of aliquots of the contents of the small intestine indicated that approximately 22,200 *T. colubriformis* worms were harbored. Numerous adolescent *Cooperia punctata* were found also, probably as a result of exposure on the contaminated pasture.

Calf 1506 (group 4) had a light, natural coccidial infection as evidenced by fairly low peaks of oocysts of *E. ellipsoidalis* on experiment days 19 to 22, with diarrhea appearing on only one day throughout the test. Weight gains (table 1) were continuous except during the fourth week.

Calf 1501 (group 4) had a moderate, natural coccidial infection (table 1) as shown by *E. ellipsoidalis* oocyst peaks between days 17 and 24, followed by low peaks of *E. zurnii* oocysts until day 29. The latter species was associated with diarrhea, but the weight gains (table 1) were not depressed.

Experiment 2.—As in experiment 1, the combination of the two kinds of parasites was more destructive than the coccidia alone, as indicated by the death of both calves in group 2 on experiment days 20 and 21, during the peak of oocyst production. By the third week the average weight loss was 3.0 lb. per calf in this group. These calves had shown diarrhea on an average of 66 per cent of the days before their deaths, as compared with 71, 34, and 23 per cent in groups 1, 3, and 4, respectively (table 2). The calves in these three groups had diarrhea on an average of 64, 61, and 6 per cent, respectively, of the remaining 14 days of the experiment. During the fifth and sixth weeks the group 3 calves made slightly smaller average gains than those in group 1, again indicating that coccidial harm had passed its peak but that the worms continued to affect the rate of gain. After the second week, the controls made consistently better average gains than any of the others.

Group 2 was the only one in experiment 2 in which an oocyst count of over 10,000 per loopful occurred (calf 1549, on one day), and the group had average oocyst counts of over 1,000 per loopful on 45.7 per cent of the days examined, as compared with 19, 25, and 10 per cent of the days for groups 1, 3, and 4, respectively. However, calves in these three groups were examined on nearly twice as many days as those in group 2, as a result of the deaths in the latter group. During the first 21 days the group 3 calves had peaks of oocysts higher than 1,000 per loopful on 37.1 per cent of the days examined, as compared with 17.7 per cent for those in group 1, despite the lower numbers of infective oocysts administered to the group 3 calves.

Calf 1537 (group 1) began to have diarrhea on experiment day 13 and it continued almost every day until day 34, even though neither mucus nor blood was observed macroscopically. By day 19, the calf was too weak to rise, even while defecating. The initial high peak was due to oocysts of *E. canadensis* on day 20, followed by those of *E. auburnensis* on day 21 and *E. zurnii* from day 28 to 30. The infections were considered moderate.

Calf 1550 (group 1) had diarrhea nearly every day from experiment day 2 until 30. The initial high peak of oocysts occurred on day 19. Oocysts of *E. canadensis* were predominant, followed by those of *E. auburnensis* on days 20 and 21 and *E. bovis* from days 22 to 24. Oocysts of *E. zurnii* were present in small numbers. During the heavy discharge of *E. bovis* oocysts, some were expelled in intact clusters, still surrounded by tissues, suggesting the appearance of oocyst clusters in the glands of the intestine, as illustrated in a previous paper.[7] One cluster, approximately 300 μ long, indicated that an entire parasitized intestinal gland probably had been extruded and had passed intact.

Calf 1539 (group 2) developed a slight diarrhea on experiment day 6; by day 12 the feces were fluid and remained that way until the calf died on day 21. At the last, feces were dysenteric. The calf was unable to rise for three days before death. In the terminal two weeks it lost 18 lb. The initial peak of oocyst discharge was due to

those of *E. alabamensis* on days 12 and 13, followed by those of *E. canadensis* and then *E. bovis*, *E. auburnensis*, and *E. zurnii*. Intestinal scrapings and sections showed that a massive coccidial attack was underway, with many endogenous stages found in every scraping made from the upper small intestine to the rectum. There were numerous petechiae in the anterior portion of the duodenum; the gallbladder was abnormally distended; and 48,000 *T. colubriformis* were recovered from the small intestine.

Calf 1549 (group 2) had diarrhea on experiment days 5, 6, 12, and 14 and then continuously until death on day 20. While the calf was moribund, stools were dysenteric. The calf was too weak to rise on the last two days. At necropsy the mucosa of the upper portion of the duodenum showed numerous petechiae. Oocysts of *E. auburnensis* were dominant in the terminal 48 ft. of the 72-ft. small intestine and also in the cecum, colon, and rectum. Histologic sections showed that the upper colon had more parasitized glands than the middle or lower colon. Oocysts of *E. alabamensis*, *E. ellipsoidalis*, and occasionally of *E. canadensis* were found in the duodenum. The coccidial infection was considered one of the greatest in the two experiments. Only 24,810 worms were recovered.

Calf 1540 (group 3) developed diarrhea on experiment day 15 and it continued until day 25, at which time oocysts ceased to be discharged. The initial high peak of oocysts was due to those of *E. auburnensis* on day 15, followed by small numbers of *E. zurnii* oocysts for four days; *E. auburnensis* were again dominant on days 20 and 21. There was a progressive weight gain each week, except during the peak of coccidial infection which occurred during the third week (table 2).

Calf 1547 (group 3) had diarrhea every day from experiment day 15 to 36. Blood was present on three days. The first peaks of oocysts were due to *E. ellipsoidalis*, probably as a result of natural infection, as discharge of oocysts of this species started on day 5 and continued until day 15, followed by those of *E. alabamensis* on day 16, *E. bukidnonensis* from day 17 to 19, *E. bovis* for three more days, and those of *E. auburnensis* terminating the peaks of discharge on day 23. This calf evidently was more heavily infected than calf 1540, as it did not gain weight until the sixth week and there was a weight loss during the fourth week (table 2).

Calf 1542 (group 4) developed a light natural coccidial infection. It had diarrhea only on experimental day 5, with blood appearing only on day 9 and mucus on day 25. The weight gains were consistent during weeks 2 to 6 (table 2).

Calf 1551 (group 4) had a moderate infection from natural coccidial contamination, as shown by peaks of *E. zurnii* oocysts on experiment day 2 and *E. ellipsoidalis* oocysts during the first week before the inoculated species in experimentally infected calves had a chance to complete their cycles. This accounts for most of the peaks of 1,000 to 10,000 oocysts shown in table 2. Diarrhea occurred on days 5 to 11, but neither blood nor mucus was seen. During weeks 2 and 3 the weights remained nearly constant, but the total weight gained during the test was good (table 2).

SUMMARY

Eight calves were allotted to four groups in each of two replicate experiments comparing the effects of Eimeria spp. given at the rate of 3,000 oocysts per pound of body weight (group 1); Eimeria spp. given at the same rate plus 70,000 larvae of *Trichostrongylus colubriformis* (group 2); or half the numbers of coccidia and larvae administered in the second group (group 3). Group 4 was composed of untreated control calves.

In both experiments the group 2 combination of Eimeria spp. and *T. colubriformis* was more destructive than the coccidia alone, with 1 calf in experiment 1 and both calves in experiment 2 dying as a result of the full dose mixed infections.

In experiment 1 the group 3 calves had diarrhea for about one third more days than those in group 1 and for nearly three times as many days as those in group 2. The calves given the reduced numbers of both parasites had high oocyst counts on a few more days than the calves given the full amounts of both. By the sixth week they also made the poorest total weight gains of the surviving calves, and both died from parasitism shortly after going onto pasture at the end of the test.

In experiment 2, by the time of the deaths of the calves in group 2 on experimental days 20 and 21, they had high daily oocyst

counts on 45 per cent of the days examined as compared with only 17 per cent in group 1 calves. Even the calves in group 3 showed the high counts on an average of 37 per cent of the days. As in the first experiment, group 3 calves made the poorest weight gains of the surviving calves by the end of six weeks.

The presence of the nematodes affecting the same general region of the intestine as the coccidia may have had a synergistic effect on the development of the coccidia.

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The Combined Effect of Infectious Bovine Rhinotracheitis Virus and *Pasteurella hemolytica* on Cattle

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THE OBJECTIVE of this experiment was to determine the effect of superimposing *Pasteurella hemolytica* infection on cattle already affected with the virus of infectious bovine rhinotracheitis (IBR). Various workers [2,4,6] have associated *Past. hemolytica* with fatal pneumonia in cattle. The virus of IBR is the etiologic agent of a specific respiratory disease of cattle.[3,5] Does this virus potentiate the action of the Pasteurella?

Palotay and Christensen [7] reported a temperature rise, clinical signs of respiratory disease, and death in one case as a result of inoculating calves intratracheally with a combination of *Past. hemolytica* and the virus of sporadic bovine encephalomyelitis. Intratracheal inoculation of either of these agents separately into calves either did not elicit any observable response or just transient evidence of disease.

MATERIALS AND METHODS

Sixteen Hereford heifers that averaged 320 lb. in weight were obtained directly from the ranch on which they were raised. There was no history of respiratory disease in these animals. Blood serums from these cattle were negative for neutralizing antibody for IBR virus. No Pasteurella sp. was obtained on culture of nasal mucus. These calves were allotted at random into four lots (I, II, III, and IV) of 4 each. Those in each lot were stanchioned in a different room of a maximum security isolation unit. The air temperature was maintained at 68 F. A preinoculation period of three weeks was allowed for the animals to adjust to this environment. During this interval, normal ranges of body temperatures and total leukocyte counts were established for each animal.

The terms, "febrile," "leukocytosis," and "leukopenia," were used to indicate deviations from these established ranges during the postinoculation period. A maintenance ration of alfalfa hay, protein pellets, and salt was provided. Drinking water was available to the calves at all times.

A 2 x 2 factorial design was used (table 1). Treatment A consisted of the inoculation of each calf in lots II and IV with *Past. hemolytica* (strain 801) culture. This culture was freshly isolated from frozen lung tissue of a calf that died of shipping fever. The source tissue had been maintained 15 months at −20 F. A 24-hour tryptose broth culture, 1 ml. per animal, was nebulized into one end of a plastic bag; the other end was secured over the muzzle of the calf. The animal then inhaled an undetermined fraction of a milliliter of this inoculum.

Treatment B (lots III and IV) consisted of the intratracheal injection of each calf with 2 ml. of a $10^{5.2}$ tissue culture infective dose$_{50}$ of IBR virus. It is apparent, then, that lot IV received both inoculums and that lot I received neither of them.

The IBR virus was administered on day zero (fig. 1). The *Past. hemolytica* culture was given three days later. There were no additional inoculations.

RESULTS

Postinoculation respiratory infections developed in the calves of lots II, III, and IV. The intensity and duration of the febrile reactions is indicated (fig. 1). Other evidences of disease coincided with the febrile period (table 2). Some individual variation in susceptibility was evident (fig. 1). All sick animals recovered without medication. None were killed for necropsy.

Three of the 4 calves in lot II were febrile within 48 hours postinoculation; 2 were moderately dyspneic at that time. A marked degree of leukocytosis occurred during the febrile rise but quickly subsided. Clinical signs in 2 calves included mucopurulent nasal discharge, anorexia, occasional coughing, a dejected attitude, and bronchial râles. The coughing and subdued attitude persisted for several days after the fever subsided in these 2 animals. *Past. hemolytica* was recovered from the nasal mucus at 15 days postinoculation. One animal in this lot did not become sick.

All calves of lot III were febrile on the third day postinoculation. The febrile reactions in these calves achieved a higher

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TABLE 1—Experimental Design

		Pasteurella hemolytica inoculum	
		0	1
IBR virus inoculum	0	(1) (Lot I)	(a) (Lot II)
	1	(b) (Lot III)	(ab) (Lot IV)

level than those in lot II and persisted longer (fig. 1). Clinical signs included anorexia, lacrimation, stringy seromucous nasal discharge, some coughing, subdued attitude, and stertorous breathing in 1 animal. A slight leukopenia was detected at 24 hours postinoculation. The IBR virus was recovered from the nasal mucus of these animals at seven days postinoculation but not at nine days.

All calves in lot IV were febrile within 48 hours postinoculation, a day sooner than those in lot III. The *Past. hemolytica* inoculum was administered to these calves on the third day following intratracheal injection of the IBR virus (fig. 1). Febrile reactions reached a higher peak and persisted for a longer interval in calves in this lot than in those in either lot II or lot III. Slight leukopenia followed the virus inoculation, but no leukocytosis followed inoculation of the bacteria in lot IV calves. This was in contrast to the marked leukocytosis in lot II animals. The clinical signs were a combination of those observed in affected animals in both of the preceding lots. Clinically, the calves in lot IV were not appreciably more sick than those of lot II, but the signs of disease were of greater duration in the former group. Convincing evidence of pneumonia of the anterior and ventral areas of the lungs was obtained on auscultation of 2 of the animals in lot IV.

Biometric analysis of the temperature data revealed statistical significance at the 5 per cent level in both main effects but not in interaction during the initial part of the febrile reaction (table 3). During the later days of the febrile period, significance per-

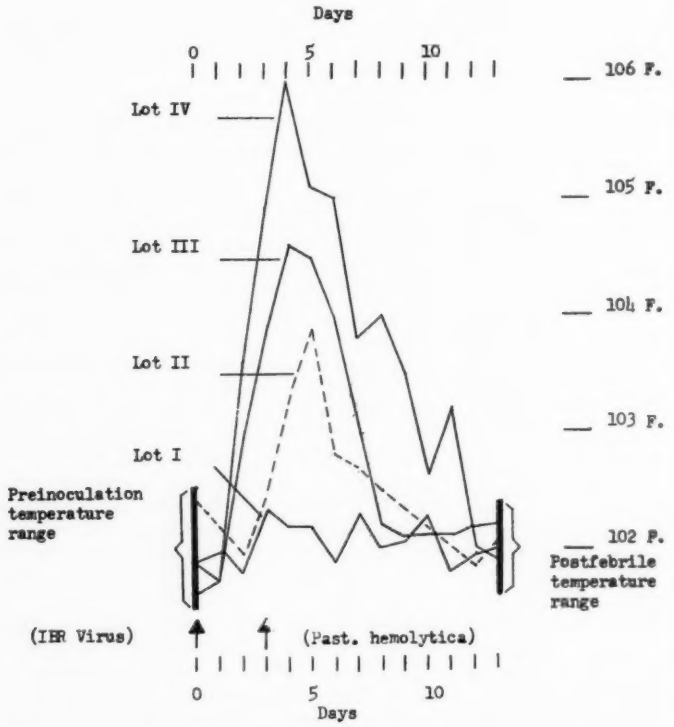

Fig. 1—Lot means of body temperatures by days.

TABLE 2—Some Evidences of Disease in Inoculated Cattle

Lot No.	Animal No.	Days of febrile reaction	Maximum body temperature (F.)	Days of leukocytosis	Days of leukopenia	Signs of pneumonia
I	1, 2, 3, 4	..	102.4 (mean)	.....	.....	.....
	5	3	105.8	2(11.4)*	.....	±**
	6	2	104.8	2(6.7)*	.....	.....
II	7	..	103.0	1(2.3)*	.....	.....
	8	2	103.8	1(4.4)*	.....	±
	9	4	104.4	.....	1(3.5)*	.....
	10	4	105.2	.....	.....	.....
III	11	6	104.6	.....	1(2.5)*	.....
	12	6	105.4	.....	2(2.2)*	.....
	13	9	106.5	.....	2(S)†	+
IV	14	9	105.6	.....	.....	+
	15	6	104.9	.....	.....	.....
	16	7	107.0	.....	2(S)	.....

* Numbers in parentheses indicate standard deviations from the mean of preinoculation total leukocyte counts. ** Dyspnea, bronchial râles, loss of sound conduction. † S = slight.

sisted in treatment A *(Past. hemolytica)*, treatment B (IBR virus) was not significant, and the mean square value of AB (interaction) increased appreciably. In other words, the analysis indicated that the Pasteurella infection was responsible for the elevation of body temperature during the latter part of the febrile reaction.

DISCUSSION

Inspection of the febrile curve for lot IV calves (fig. 1) reveals a mean value of nearly 105 F. on the third day postinoculation (of virus) as compared to a value of 103.9 F. for lot III calves on the same day. This level in lot IV animals was reached prior to the administration of the *Past. hemolytica* inoculum. It seems possible then that the curve for this lot could have reached the peak value of 106 F. a day later without the addition of the bacterial inoculum.

The results of the analysis of the temperature data support the belief that each infectious agent exerted its effect independently of the other during the crucial febrile reaction in the animals of lot IV. A comparable intensity of the clinical signs in calves of lots II and IV also supports this view. The approach of interaction toward statistical significance later in the course of the febrile period and the persistence of statistical significance in treatment A, however, may indicate some enhancement of the *Past. hemolytica* infection in the presence of the IBR virus infection. The extended course of the disease and the evidence of pneumonia in 2 animals also supports this latter view.

The marked leukocytosis that followed the administration of the *Past. hemolytica* inoculum in the calves of lot II did not occur following its administration in lot IV calves. Since lot IV calves received IBR virus as an additional treatment, it is logical to conclude that the suppression of leukocytosis was due to the action of the virus. If this is true, then a mechanism is indicated for enhancement of the bacterial infection by the virus, *i.e.*, decreased efficiency of the cellular defense mechanism.

In considering the effects of the infectious agents on the calves in this experiment, it must be remembered that environmental stresses were minimized. Individual variation in the susceptibility of animals within lots could, then, be attributable to genetic factors. (Genetic variation in susceptibility of cattle to shipping fever is seen under field conditions. The morbidity rate in natural outbreaks seldom exceeds 20 per cent [1].)

The combined inoculation of IBR virus and *Past. hemolytica* caused a more pro-

TABLE 3—Analysis of Variance of Body Temperature Data

Days post-inoculation (of virus)	Source of variation	Degrees of freedom	Mean square
0	Treatment A (*Past. hemolytica*)	1	0.64
	Treatment B (IBR virus)	1	0.36
	A x B	1	0.01
	Residual (error)	12	0.26
4	A	1	6.38*
	B	1	26.27**
	A x B	1	0.07
	Residual	12	1.16
9	A	1	2.89*
	B	1	1.44
	A x B	1	1.21
	Residual	12	.41

* Significant at 0.05. ** Significant at 0.01.

found effect in calves than did either of these agents separately. These findings are in agreement with those obtained by Palotay and Christensen,[7] who used the virus of sporadic bovine encephalomyelitis and *Past. hemolytica*.

Shipping fever and IBR were recognized as distinct disease entities only within the past eight years. Feedlot cattle affected with IBR commonly acquired secondary bacterial infections that resulted in fatal pneumonia. According to Scheel,[8] the routine immunization of feedlot cattle against IBR in recent years has dramatically reduced the mortality rate due to fibrinous pneumonia. This observation indicates that some of the "shipping fever" seen in Colorado feedlots in years past was probably due to secondary bacterial infection following IBR. The results of this experiment indicate that infection with IBR virus does predispose cattle to serious infection with *Past. hemolytica*, a bacterium commonly associated with fibrinous pneumonia of cattle.

SUMMARY AND CONCLUSION

1) Young cattle that were maintained in a controlled environment were inoculated with either infectious bovine rhinotracheitis (IBR) virus or *Pasteurella hemolytica* culture, or with both of these agents. Respiratory tract infections developed in each of these groups.

2) The intensity of clinical signs in animals given both inoculums was not appreciably greater than in those given them singly. The duration of sickness was greater, however, in the group given both inoculums. Evidence of pneumonia was obtained on auscultation of the lungs of 2 of these animals.

3) It is concluded that infection with IBR virus contributed to the seriousness of infection due to *Past. hemolytica* in cattle, although no profound potentiating effect occurred.

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Pulmonary Hypertension and Right Ventricular Hypertrophy in Cattle at High Altitude

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HYPOXIA has been demonstrated to cause a rise in the pulmonary arterial blood pressures of various species. Von Euler and Liljestrand [2] first observed this change in the cat. Lewis and Gorlin [5] observed the same phenomenon in dogs breathing hypoxic mixtures. Stroud and Conn [9] made similar observations in the dog and interpreted such changes to be due to vasoconstriction of the pulmonary vascular bed. Rotta *et al.*[8] measured the pulmonary arterial pressures of human beings residing at sea level and at an altitude of 14,900 feet. Their studies disclosed a significant increase in pulmonary arterial pressures at the higher altitude.

Alexander and Jensen [1] demonstrated a statistically significant right ventricular hypertrophy both in clinical cases of bovine high mountain (brisket) disease and in experimental cattle residing at high altitudes. They postulated chronic hypoxia to be an etiologic factor in the development of the hypertrophy and in the pathogenesis of high mountain disease by increasing pulmonary vascular resistance and work load upon the right side of the heart.

The following experiment was carried out to study the effect of residence at high altitude upon the pulmonary arterial pressure in cattle, and to determine the relationship of pulmonary arterial pressure to the right ventricular mass.

MATERIAL AND METHODS

Twenty yearling Hereford steers of uniform breeding were obtained from a single ranch in eastern Colorado, at an elevation of 3,600 feet. The cattle were randomized by weight into two lots of 10 animals each. Animals in one lot served as controls and were maintained at an altitude of 5,000 feet, while those in the second lot were placed at an elevation of 10,000 feet. Both lots were confined in corrals and fed identical rations (alfalfa hay and concentrate pellets) grown and prepared at low elevation.

Pulmonary arterial pressures were measured by using a vascular catheterization technique. The animals were confined in a squeeze chute and blindfolded. A 150-cm., No. 6 Cournand cardiac catheter was inserted through a 12-gauge needle into the left jugular vein. The catheter was advanced through the right side of the heart into the pulmonary artery. The characteristics of the pulse wave determined the location of the catheter. Pressures were recorded by means of an E and M, P-500 strain-gauge transducer connected to a direct-writing, multichannel, electronic recorder. The system was calibrated with a mercury manometer. Mean pulmonary arterial pressures were determined by planimetering the resulting tracings.

During the experiment, pulmonary arterial pressure measurements were made four times on each animal (table 1). The initial measurements on all animals were made at an elevation of 5,000 feet. The subsequent three determinations were made during the second, fourth, and sixth months of the experiment while the animals were residing at their respective treatment altitudes. The duration of the experiment was seven months, from June, 1958, through January, 1959. At time IV, additional pulmonary arterial pressure measurements were made on animals 3 and 12, during a ten-minute period while each animal was breathing 100 per cent oxygen through a mask.

At termination of the experiment the animals were slaughtered and observed for gross pathologic changes. The hearts were divided into ventricular components and weighed; the ratio of each ventricular component to the total ventricular weight was calculated.

Results of the pulmonary arterial pressure measurements revealed a natural separation within the high-altitude group, commencing with time II. Based on this observation, this lot was subdivided into Group A (animals 2, 3, 17, 19) and Group B (animals 4, 5, 12, 13, 15, and 16). In statistical analyses of the data, comparisons were made between the control lot and the high-altitude lot; the control lot and group A; control lot and group B; and between groups A and B (table 2).

RESULTS

Data obtained from the pulmonary arterial pressure measurements and statistical analyses are presented (tables 1, 2;

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fig. 1). Initial measurements of the pulmonary arterial pressure made on all animals at low altitude (5,000 feet) showed no statistical difference between the low and high altitude treatment groups, with a mean of 28 mm. of Hg. in the control lot and a mean of 26.7 mm. of Hg. in the high altitude lot. Subsequent measurements of the control group at times II, III, and IV showed no statistical difference in the mean pulmonary arterial pressures when subjected to analysis of variance.

Pulmonary arterial pressures of the high-altitude cattle at time II (7 weeks) showed a statistically significant elevation ($P < 0.005$) when compared with the control group. A subdivision was apparent within the high-altitude group at this time; animals 2, 3, 17, and 19 (group A) showed distinctly higher pulmonary arterial pressures than the remaining animals (group B). Animal 19 had a pressure of 77 mm. of Hg., which was approximately three times the pressure recorded at low altitude in that steer. Animals 2, 3, and 17 had pulmonary arterial pressures of 51, 65, and 55 mm. of Hg., respectively, which were more than twice as great as their initial values at low altitude. A significant elevation of the pressure was found ($P < 0.001$) when this more markedly hypertensive group was compared with both the control group and the remaining animals of the high-altitude lot. Pressures of the less hypertensive steers within the high-altitude lot averaged 36.3 mm. of Hg. and were significantly elevated over those of the low-altitude controls ($P < 0.005$).

Pulmonary arterial pressure measurements made at time III (17 weeks) continued to show a significant difference between the high- and low-altitude groups. When compared with measurements made at time II, the average pressure of animals 2, 3, 17, and 19 was markedly elevated with a mean of 83.2 mm. of Hg.; the average pressure of the remaining 6 animals in the high-altitude group was 38.8 mm. of Hg., which indicated a moderate increase (fig. 1).

The mean pulmonary arterial pressure of animal 19 was 107 mm. of Hg. at time III. During the 21st week at high altitude, this steer developed subcutaneous edema, ascites, a jugular pulse, and diarrhea. The mean pulmonary arterial pressure at this time was 104 mm. of Hg. and is included at time IV in tables 1 and 2 and in figure 1. The steer showed marked weakness and depression and was moved to lower altitude for euthanasia. Necropsy findings in this animal were severe subcutaneous edema, dilatation and hypertrophy of the right ventricle, and chronic passive congestion of the liver and other viscera.

Pressures recorded at time IV (tables 1, 2; fig. 1) were obtained from the surviving animals during the sixth month of the experiment but include the terminal pressure obtained during the fifth month on steer 19. Analysis of data obtained at time IV again showed significantly elevated pressures in the high-altitude steers ($P < 0.05$). Animals 2, 3, 17, and 19 had pressures which were significantly higher (mean, 89.7 mm. of Hg.) than those of the other animals in the high-altitude lot (mean, 45.1 mm. of Hg.). These less hypertensive animals of the high-altitude group had pressures which were significantly elevated over those of the control lot (mean, 27.4 mm. of Hg.).

Pulmonary arterial pressure measurements obtained while administering oxygen to animals 3 and 12 are presented (fig. 2).

Cardiac ventricular ratios obtained from both groups are presented (table 3) and these data are analyzed (table 4). A significant increase ($P < 0.005$) in the weight ratio of the right ventricle to the total ventricular mass (RV/T) was present in the

TABLE 1—Mean Pulmonary Arterial Pressures, in Millimeters of Mercury

Animal No.	Times I	II	III	IV
5,000-FT. ELEVATION				
1	26	23	21	29
6	37	31	29	25
7	29	21	31	22
8	30	32	31	32
9	28	31	23	23
10	30	22	21	22
11	30	30	30	28
14	24	26	20	29
18	26	30	25	29
20	20	30	19	35
Mean	28.0	27.6	25.0	27.4
Standard deviation	4.5	4.3	4.8	4.4
10,000-FT. ELEVATION				
2	25	51	79	92
3	27	65	86	109
4	19	43	40	43
5	26	35	38	49
12	28	33	42	48
13	32	39	41	41
15	34	34	36	48
16	22	34	36	42
17	27	55	61	54
19	27	77	107	104
Mean	26.7	46.6	46.6	63.0
Standard deviation	4.4	15.1	23.0	28.8

high-altitude group as compared to the low-altitude control lot. Hearts from the animals with severe pulmonary hypertension showed a significant elevation in the RV/T ratio when compared with both the control lot ($P < 0.001$) and with the remaining animals in the high-altitude group ($P < 0.001$). Hearts from animals in the high-altitude lot which exhibited moderate pulmonary hypertension showed significant elevation in the RV/T ratio when compared with those from the control lot ($P < 0.001$). Relationship between the final mean pulmonary arterial pressure and the RV/T ratio of all animals is shown (fig. 3). Linear regression of these data showed a correlation coefficient of 0.952 with a probability of < 0.001.

Necropsy of animal 3 showed marked dilatation and hypertrophy of the right ventricle accompanied by chronic passive congestion of the liver.

DISCUSSION

The initial measurements of pulmonary arterial pressure made at low altitude on all animals showed marked uniformity. This uniformity of pressure was maintained by the control lot throughout the entire experiment (standard deviation of 4.3 to 4.8 mm. of Hg.). The consistency of these measurements suggested that a value of 25 to 28 mm. of mercury represented the normal mean pulmonary arterial pressure of cattle at an elevation of 5,000 feet.

The pulmonary hypertension which developed in the cattle residing at a high altitude is similar to the findings of Rotta *et al.* for man.[8] Elevation of the pulmonary arterial pressure was apparent at time of the second measurement after seven weeks at an altitude of 10,000 feet. The hypertensive response within this group did not, however, appear uniform at this time. The 4 steers (subgroup A) with highest pulmonary arterial pressures were the same animals which later developed severe pulmonary hypertension. Cardiac decompensation subsequently occurred in 2 of these animals. Statistical differences in pulmonary pressures between subgroup A and subgroup B and between these subgroups and the control animals indicated that separation was justified.

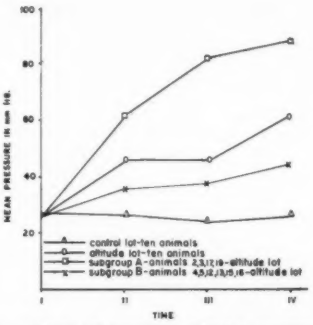

Fig. 1—Mean pulmonary arterial pressures of cattle at low and high altitudes.

TABLE 2—Statistical Analysis of Mean Pulmonary Pressures

Time	Lot	Mean	Standard deviation	Comparing	Value of T	Probability
I	Control	28.0*	4.5	Control vs. altitude	0.660	.....
	Altitude-all	26.7	4.4	.....	.....	.....
	A**	26.5	1.0	Control vs. A	0.640	
	B**	26.8	5.9	Control vs. B	0.440	
				A vs. B	0.110	
II	Control	27.6*	4.3	Control vs. altitude	3.830	<0.005
	Altitude-all	46.6	15.1	.....	.....	
	A	62.0	11.0	Control vs. A	8.480	<0.001
	B	36.3	4.1	Control vs. B	4.060	<0.005
				A vs. B	5.090	<0.001
III	Control	25.0*	4.8	Control vs. altitude	2.900	<0.01
	Altitude-all	46.6	23.0	.....	.....	
	A	83.2	19.2	Control vs. A	9.410	<0.001
	B	38.8	2.86	Control vs. B	6.320	<0.001
				A vs. B	5.750	<0.001
IV	Control	27.4*	4.4	Control vs. altitude	2.770	<0.025
	Altitude-all†	63.0	28.8	.....	.....	
	A†	89.7	25.0	Control vs. A	8.060	<0.001
	B	45.1	4.1	Control vs. B	8.100	<0.001
				A vs. B	4.410	<0.005

* No statistical difference between these measurements.
** A = high-altitude animals 2, 3, 17, and 19; B = high-altitude animals 4, 5, 12, 15, and 16.
† Includes terminal measurement on animal 19.

TABLE 3—Ventricular Ratios

Animal	Ventricular ratios		
	RV/T	LV/T	S/T
5,000-FT. ELEVATION			
1	0.247	0.460	0.293
6	0.254	0.496	0.250
7	0.269	0.459	0.272
8	0.240	0.477	0.283
9	0.231	0.466	0.303
10	0.253	0.471	0.276
11	0.231	0.458	0.311
14	0.236	0.510	0.254
18	0.235	0.511	0.254
20	0.232	0.504	0.264
Mean	0.2428	0.4812	0.2760
Standard deviation	0.0127	0.0218	0.0213
10,000-FT. ELEVATION			
2	0.382	0.354	0.264
3	0.420	0.329	0.251
4	0.272	0.441	0.287
5	0.261	0.462	0.277
12	0.297	0.435	0.268
13	0.275	0.459	0.266
15	0.271	0.455	0.274
16	0.281	0.427	0.292
17	0.335	0.411	0.254
19	0.448	0.307	0.245
Mean	0.3242	0.4080	0.2678
Standard deviation	0.0687	0.0570	0.0152

RV/T = ratio of the right ventricular weight to the total ventricular weight; LV/T = ratio of the left ventricular weight to the total ventricular weight; S/T = ratio of the weight of septum to the total ventricular weight.

Pressure measurements of the high-altitude animals which developed only moderate pulmonary hypertension (subgroup B) had the same relative standard deviations at times II, III, and IV as the control animals. This similarity of standard deviations indicated that the hypertension within this group was within normal limits and probably represented a normal physiologic response to the hypoxic environment. This low standard deviation was maintained by subgroup B animals, even though their mean pressure continued to rise throughout the experiment, and was significantly elevated over that of the controls. At no time during the experiment did the animals in subgroup B show evidence of cardiac decomposition or other clinical signs which would distinguish them from the control animals.

Signs and lesions observed in animal 19 were typical of those seen in high mountain disease of cattle.[1,3,4,6,7] The right ventricular hypertrophy and dilatation with chronic passive congestion of the liver observed at necropsy in animal 3 were characteristic of early cardiac decompensation in cattle. Antemortem observation of animal 3 after time III suggested the presence of subcutaneous edema in the intermandibular space, but this change was not found at necropsy.

Cardiac ventricular ratio data obtained from the control animals compared favorably in respect to both actual value and standard deviations with that previously reported for cattle residing at an altitude of 5,000 feet.[1] Ventricular ratios from the less hypertensive cattle at high altitude are in agreement with those previously obtained from normal steers residing at a high altitude.[1] Animal 19, which showed clinical signs of congestive failure, had the heart with the highest RV/T ratio. Animal 3, which showed early lesions of cardiac decompensation, had the heart with the next highest RV/T ratio. The RV/T ratios from the remaining 2 animals of the markedly hypertensive group were proportional to the respective mean pulmonary arterial pressure and larger than the ratios of the less hypertensive group. The strong posi-

TABLE 4—Statistical Analysis of Ventricular Ratios

Ventricular ratio	Lot	Mean	Standard deviation	Comparing	Value of t	Probability
RV/T	Control	0.2428	0.0127	Control vs. altitude	3.682	<0.005
	Altitude-all	0.3242	0.0687	.....	.....	
	A	0.3962	0.0492	Control vs. A	9.622	<0.001
	B	0.2761	0.0130	Control vs. B	5.054	<0.001
				A vs. B	5.839	<0.001
LV/T	Control	0.4812	0.0218	Control vs. altitude	3.790	<0.005
	Altitude-all	0.4080	0.0570	.....	.....	
	A	0.3502	0.0451	Control vs. A	7.523	<0.001
	B	0.4464	0.0160	Control vs. B	3.368	<0.005
				A vs. B	4.910	<0.005
S/T	Control	0.2760	0.0213	Control vs. altitude	0.989	
	Altitude-all	0.2678	0.0152	.....	.....	
	A	0.2535	0.0079	Control vs. A	2.012	
	B	0.2773	0.0109	Control vs. B	0.141	
				A vs. B	3.739	<0.01

A = High-altitude animals 2, 3, 17, and 19; B = High-altitude animals 4, 5, 12, 13, 15, and 16.

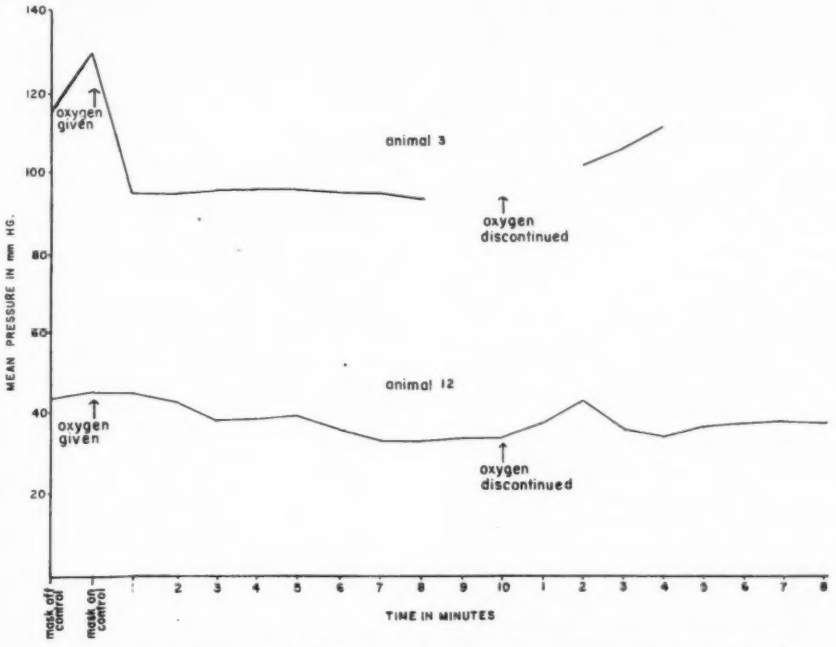

Fig. 2—Mean pulmonary arterial pressure of animals 3 and 12, breathing pure oxygen (time IV).

tive correlation ($r = 0.952$) between pulmonary arterial pressure and right ventricular mass suggested that the ventricular hypertrophy was a result of increased pulmonary vascular resistance. These observations and statistical differences further justified subdivision of the high-altitude group.

The hypertensive response at high altitude is believed to be due to a chronic hypoxia resulting from the low partial pressure of oxygen.[8] Experimental design of this study controlled a number of factors, including feed and exercise. The only experimental treatment was resident altitude. While definite conclusions cannot be based on data obtained from 2 animals, the decrease in the pulmonary arterial pressures observed in animals 3 and 12 while they were breathing pure oxygen supports the interpretation of chronic hypoxia as a cause of this pulmonary hypertension. While breathing 100 per cent oxygen, the pulmonary arterial pressure of the moderately hypertensive animal (No. 12) decreased to a value approximating the upper limits in the control animals. The reduction of the pulmonary arterial pressure of the severely hypertensive steer (No. 3) during the same treatment was greater; however, the pres-

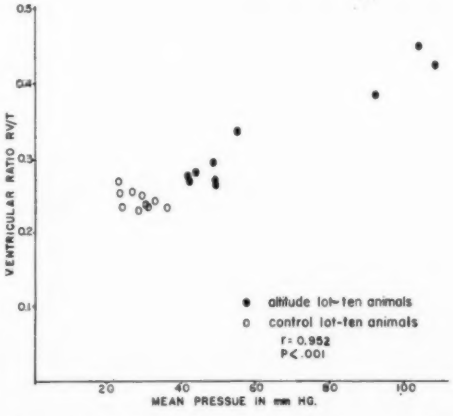

Fig. 3—Comparison of mean pulmonary arterial pressure with the weight ratio of the right ventricle to the total ventricular mass (time IV).

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sure did not decrease to normal values. Similar studies in man using only 35 per cent oxygen showed no reduction in pulmonary arterial pressures.[8]

The magnitude of the hypertensive response in cattle is apparently greater than that of man.[8] It is therefore supposed that the bovine species is particularly sensitive to a hypoxic environment. This hypothesis agrees with the fact that only in the bovine animal is congestive right ventricular hypertrophy commonly seen at altitudes of 7,000 to 10,000 feet. It is considered that these experimental findings further support the role of hypoxia in the pathogenesis of high mountain disease in cattle.

SUMMARY

1) A designed experiment was conducted to study the effect of residence at high altitude upon the pulmonary arterial pressure of cattle and to determine the relationship of pulmonary arterial pressure to right ventricular mass.

2) Significant elevation of the pulmonary arterial pressure was observed in the animals residing at a high altitude. Pulmonary arterial pressure of the control animals at a low elevation showed no significant change.

3) Four of the 10 animals at the high altitude developed marked pulmonary hypertension. One of these 4 steers developed clinical signs and postmortem lesions typical of high mountain disease in cattle. At necropsy, a second animal within this markedly hypertensive group had lesions of early cardiac decompensation.

4) Cardiac ventricular ratios revealed a significant right ventricular hypertrophy in the animals at the high altitude. A strong positive correlation was demonstrated between mean pulmonary arterial pressure and right ventricular mass.

5) The pulmonary hypertensive response at the high altitude is postulated to be due to chronic hypoxia. Reduction of pulmonary arterial pressures of 2 hypertensive animals upon administration of 100 per cent oxygen lends support to this hypothesis.

6) Results of this study further support the role of hypoxia in the pathogenesis of high mountain disease of cattle.

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Cannulization of the Bovine Pancreatic Duct

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THIS REPORT describes a technique for the collection of bovine pancreatic juice without contamination or activation of the pancreatic enzymes, and apparently with minimal change in the physiologic state of the experimental animal. It was developed in response to a request from the Department of Biochemistry, School of Medicine, University of Washington, for a supply of uncontaminated bovine pancreatic juice. It was recognized that if a technique could be developed whereby the animal could be maintained over an extended period of time, additional studies could be conducted on pancreatic secretions.

REVIEW OF LITERATURE

Although many methods are described for the collection of pancreatic juice in various animals, all were unsuitable for one or more reasons. Pavlov, Heidenhain, Babkin, Inlow, Dragstedt and McCaughan, all cited by Thomas,[1] have described various techniques for the transplantation of the pancreatic duct to the skin of the abdomen. However, all of these techniques have one or more of the following disadvantages: (1) difficulty of preparation; (2) possibility of destruction of normal pancreatic function; (3) activation of pancreatic enzymes from exposure to intestinal mucosa; (4) irritation of skin and subcutaneous tissue from activated trypsin; (5) disturbance of digestion from loss of pancreatic juice.

In 1941, Scott [2] reported a modification of Tuckerman's method of cannulating the pancreatic duct, in which a duodenal fistula in a dog was fitted with a metal tube having its inner end opposite the opening of the pancreatic duct. Pure, unactivated juice was collected by cannulating the pancreatic duct with a glass tube inserted through the duodenal fistula. The cannula was fitted with a screw cap to prevent loss of intestinal contents when not collecting pancreatic secretions. This method allows the collection of unactivated juice and, since there is no loss of pancreatic juice in the intervals between collections, acid-base balance and digestion should remain normal. The principal objections to this method were: (1) possibility of introducing infection into the pancreas and contaminating the pancreatic juice with bacteria picked up on the end of the cannula during the process of insertion; (2) the presence of the cannula across the lumen of the gut would act as a mechanical barrier to the passage of intestinal contents.

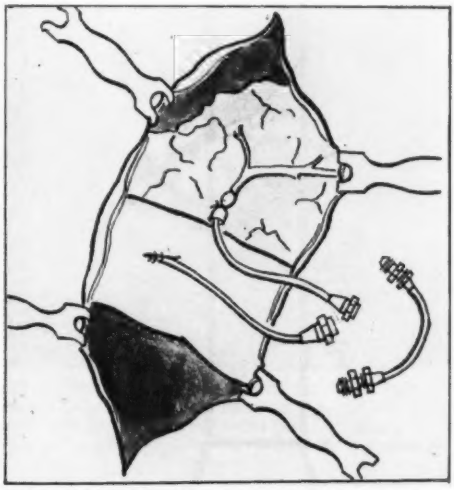

Fig. 1—Cannulae from pancreatic duct and duodenum with connecting link.

Because of these disadvantages of established techniques for cannulating the pancreatic duct, a new technique was worked out for the collection of uncontaminated, unactivated bovine pancreatic juice.

MATERIALS AND METHODS

The surgical and collection techniques were developed on 4 male Guernsey and Holstein-Friesian calves, 40 days old. They were later applied to 6 steers of these breeds, 4 to 16 months old and weighing approximately 375 to 500 lb. Feed was withheld from these animals for 12 to 14 hours prior to surgery.

Two cannulae, one from the pancreas and one to the duodenum, were exteriorized through an abdominal incision and were connected by means of brass fittings to a connecting link (fig. 1). This arrangement of cannulae permitted the collection of juice when desired or the return of juice to the duodenum in the intervals between collections.

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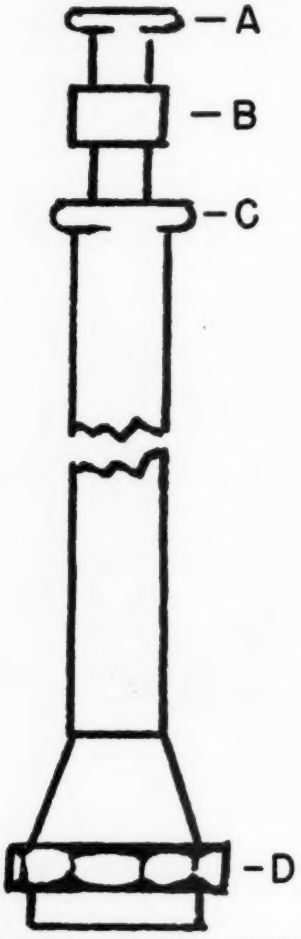

Fig. 2—Pancreatic cannula showing (A) flared end of cannula which can be inserted into duct; (B) cuff on cannula; (C) flared end of guard tube; (D) adapter on end of cannula.

The pancreatic and duodenal cannulae and the connecting link were made from polyethylene tubing,* with the addition of brass unions and nuts.

The pancreatic cannula had an inside diameter of 0.070 inch and an outside diameter of 0.110 inch; the length depending on the size of the experimental animal. Ideally, upon closure of the abdominal incision the end of the cannula should just protrude through the incision and the brass fitting lie close to the skin. Cannulae approximately 4 inches long were satisfactory for 400-lb. steers. It is advisable to have two or three cannulae of varying lengths prepared so that the surgeon can select the one desired.

The tube was cut longer than the final desired length. One end was flared slightly by the careful application of low heat with a small Bunsen burner. A brass fitting was then slipped over the tube so

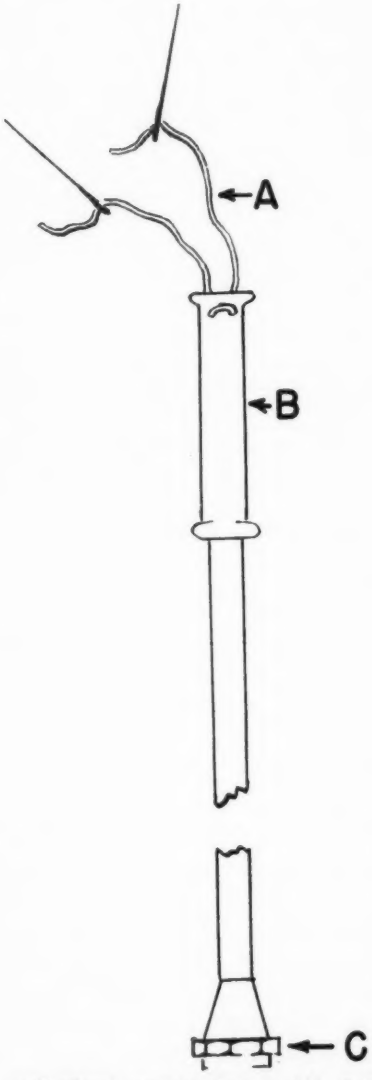

Fig. 3—Duodenal cannula showing (A) double-armed sutures; (B) 1-inch cuff on end of cannula; (C) adapter on end of cannula.

* Polyethylene tubing, medical formulation PHF, manufactured by Clay Adams Inc., New York, N.Y.

that the flared end was seated in the bevel of the coupling.

A second length of polyethylene tubing with an internal diameter of 0.115 inch and an outside diameter of 0.147 inch was also flared and then cut 7/16 of an inch shorter than the first tube. This was drawn over the cannula so that the unflared end fitted snugly against the shoulder of the brass fitting. This was most easily accomplished by placing a suture through the end of the inside tube, dropping the suture through the outside tube, and exerting traction on the inside tube while an assistant worked the two tubes together from the other end. The second tube, or "guard tube," served to double the walls of the cannula and to increase its resistance to kinking. In addition, the flare at the internal end served as an anchor for one of the duct ligatures.

Fig. 4—Connecting cannula with fittings.

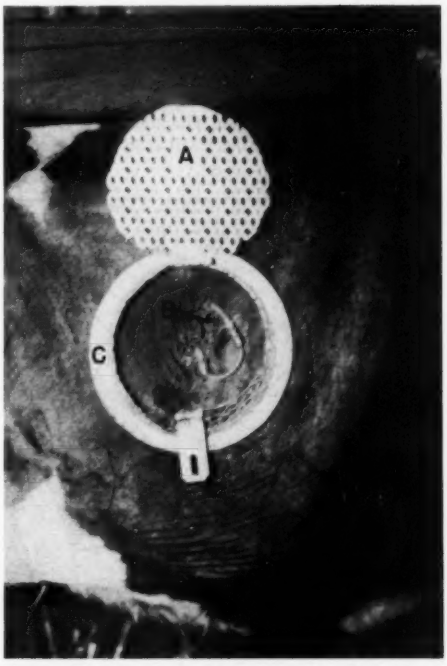

Fig. 5—Metal protective covering sutured to side of animal to protect exposed cannulae. (A) Lid of protective covering; (B) exposed ends of cannula; (C) base of protective covering with hasp on ventral aspect.

The end of the internal tube was cut off so that it protruded approximately 5/16 of an inch beyond the external tube. A 3/32-inch long cuff made of the same size tubing as the guard tube was then slipped over the internal tube and temporarily butted up against the external guard tube. The end of the internal tube was then carefully heated to produce a flare of sufficient size to securely hold the cuff from slipping off. The process of flaring shortened the internal tube so that it protruded from the outer tube approximately 3/16 inch. All sharp edges of the flares were rounded off smooth with a fine emery cloth (fig. 2).

The duodenal cannula was similarly constructed. Its length was determined by the size of the experimental animal and the angle of approach to the pancreas. A 5-inch cannula served satisfactorily for a 400-lb. steer. Again, it was advisable to prepare two or three tubes of various lengths. After cutting the two tubes, a 1-inch long cuff, flared at both ends, was constructed from polyethylene tubing with an internal diameter of 0.157 inch and an external diameter of 0.235 inch.

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A 16-inch mattress suture with straight cutting-edge needles at each end was placed through the distal end of the cuff and the cuff was then slipped over the end of the cannula. After pushing the cuff sufficiently far up to be out of the way, the ends of the double-walled cannula were simultaneously flared. Following flaring, all flared edges

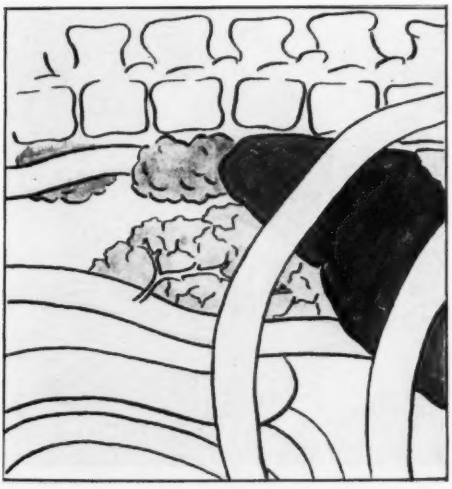

Fig. 6—Shows relative position of pancreas, duct, and duodenum.

were smoothed and rounded with emery cloth (fig. 3).

The connecting piece was prepared from a length of tubing with an internal diameter of 0.070 inch, and an external diameter of 0.110 inch. This was flared at both ends after first slipping on two brass fittings (fig. 4).

Since the animal was confined in a stanchion only during the periods of actual collection and placed in a pasture between successive experiments, a cover (fig. 5) constructed of expanded aluminum was devised to protect the exposed ends of the cannulae and the connecting link. This protective cover was shaped like an inverted pan, 1⅜-inches deep with a top diameter of 7 inches and a bottom diameter of 5 inches. A ¾-inch flange was welded around the base of the protective covering, and to this was cemented a ⅜-inch-wide, ⅜-inch-thick strip of foam rubber. Holes drilled at regular intervals around the circumference of the flange facilitated suturing the protective cover to the animal. A lid constructed of the same expanded material was fastened to the top by means of a hinge, and it was provided with a hasp to facilitate closure.

The collection equipment consisted of either 50-ml. or 125-ml. Erlenmeyer flasks provided with a two-hole rubber stopper which was fitted with two short lengths of glass tubing (fig. 9). One tube served as an air vent and was bent into a U shape at the external end. The other was a straight tube of sufficient internal diameter to just allow the insertion of a length of polyethylene tubing with an outside diameter of 0.067 inch.

During collection, the collection flask was placed in an ice container constructed from the bottom 4½ inches of a screw-capped, polyethylene quart bottle. This container was fastened in place with small spring snaps attached to the ventral side of the protective cover. These were snapped into two small rings bolted to the container ¾ inch from the top and 3 inches apart. Holes were drilled in the bottom of the container to allow the water from the melting ice to drain.

Surgery.—The animals were either restrained in lateral recumbency to minimize movement or were placed, standing, in a squeeze chute. Anesthesia is a matter of preference; in our experience, an inverted L-shaped nerve block, using 4 per cent procaine, proved most satisfactory.

After routine clipping, scrubbing, and draping, an incision was made through the skin of the right abdominal wall, 1 inch posterior to the thirteenth rib, starting approximately 3 inches from the transverse processes of the vertebra and extending ventrally, following the contour of the rib for approximately 8 inches. Following hemostasis and application of skin drapes, the muscle and fascia were incised. It is imperative that good hemostasis is effected before incising the peritoneum. The peritoneum was then incised, using blunt-pointed scissors. The incision was dilated with a Balfour abdominal retractor.

The duodenum and pancreas will generally be found directly beneath the abdominal incision (fig. 1, 6). By carefully applying traction to the segment of the duodenum to which the pancreas is attached, both the duodenum and the pancreas were lifted through the incision and held in place by an assistant. Care was taken not to traumatize the tissue during the exteriorization of the pancreas or during the subsequent exploration for the duct, since any hemorrhage interferes with locating the duct. The duct usually leaves the pancreas near the caudal end of the right lobe, which is in close proximity to the duodenum and can be located by grasping the duodenum in the left hand, exerting light traction, and palpating the tissues between the duodenum and pancreas with the right hand. The duct feels like a small cord and, when stretched over a finger, appears as a white fibrous band. However, it is not always visible through the overlying tissue, particularly if hemorrhage is encountered. The duct must be cleared of as much adjacent tissue as possible. Care must be exercized not to disturb the blood vessels in this area because the field is small and any hemorrhage interferes with the placing of the cannula; also, if blood is allowed to enter the pancreatic duct, it may activate some of the pancreatic secretion and set up a fibrosis of the duct.

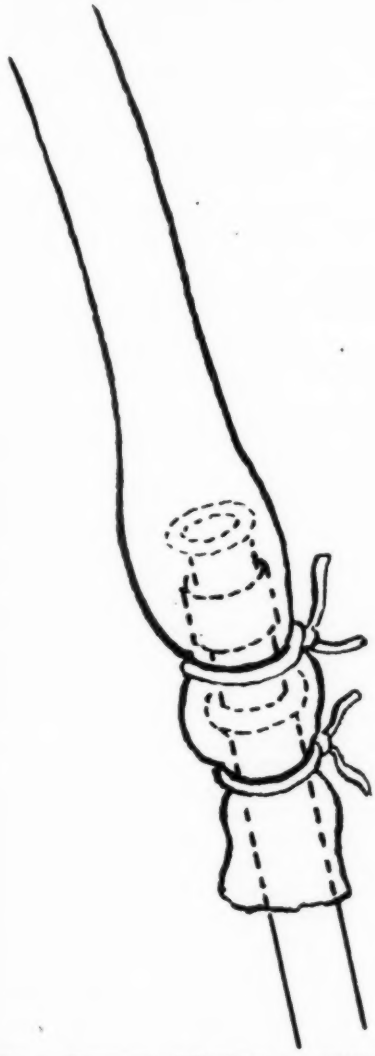

Fig. 7—Placement of ligatures on pancreatic cannula.

When the duct was isolated, two silk ligatures (size 0) were placed under the duct and tagged with mosquito forceps. A Kelly hemostatic forceps was then placed under the duct. By elevation of the forceps, the duct was slightly tensed and supported while it was incised. A ¼-inch, longitudinal incision was carefully made through the wall of the duct, the incised edges were grasped with mosquito forceps, and the wound dilated. The use of a small suction tube prevented blood from entering the duct. The end of the pancreatic cannula was then inserted into the duct toward the pancreas, but not beyond the border of the pancreas. One of the previously placed silk ligatures was then tied in so that it was seated just behind the cuff on the end of the cannulae (fig. 7); the second ligature was tied just behind the flare on the guard tube.

The duodenum was cannulated by making a small stab wound close to the entrance of the pancreatic duct. The 2-inch taper-point needles attached to the sutures of the intestinal cannula were then inserted through the stab wound 1½ inches into the lumen of the gut and forced out through the wall of the gut. By exerting traction on the sutures, the cannula was pulled into the lumen of the intestine. The needles were then removed and the sutures tied to anchor the cannula. The stab wound was closed with a Cushing stitch, using No. 0 chromic gut (fig. 8).

The free ends of the cannula were exteriorized through the abdominal wound which was closed routinely, using a continuous suture of No. 1 chromic gut for internal sutures and interrupted No. 32 stainless steel sutures in the skin. During closure of the abdominal wound, care was taken to see that the pancreatic cannula was not kinked or that it did not leave the pancreas at an acute angle.

The two cannulae were then connected by the connecting link, and the protective covering was

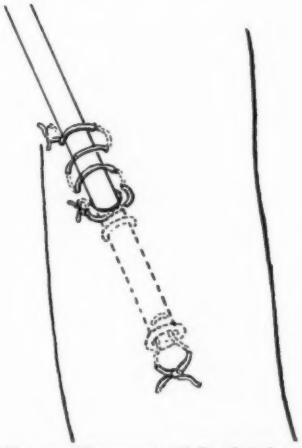

Fig. 8—Placement of duodenal cannula.

sutured to the skin with No. 26 stainless steel.

Collecting Procedures.—At the start of a collection period the connecting link was removed, leaving the brass union attached to the pancreatic side. The juice was allowed to flow for one or two minutes without collection. During this time, the ice container with the Erlenmeyer collection flask

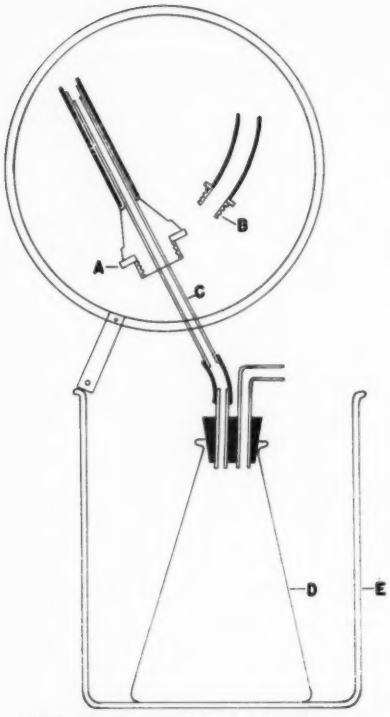

Fig. 9—Diagrammatic sketch of collecting apparatus. (A) Adapter on pancreatic cannula; (B) adapter on duodenal cannula; (C) polyethylene connecting tube inserted into pancreatic cannula and receiving tube of collecting flask; (D) collecting flask; (E) ice container attached to protective covering.

embedded in ice was attached to the protective covering. The end of the brass union on the pancreatic cannula was wiped off with alcohol and a piece of polyethylene tubing (inside diameter 0.047, outside diameter 0.067), approximately 8 inches long, was inserted through the brass fittings up into the pancreatic cannula a sufficient distance to prevent leakage and to prevent the pancreatic secretion from coming in contact with the metal on the end of the cannula. The other end of the tube was passed through one of the holes in the protective covering and inserted into the glass receiving arm of the Erlenmeyer flask. The lid was closed and held in place with a spring snap. Collection was carried out as long as desired; it was found that the ice will last approximately two hours under average conditions.

At the end of a collection period, the tube connecting the pancreatic cannula and the collection flask was disconnected from the flask. This prevented the leakage of pancreatic juice onto the animal while the ice container and flask were being removed from the protective covering. The tube was then withdrawn from the pancreatic cannula. The connecting link was attached to the pancreatic end first since there may have been a regurgitation of intestinal contents through the duodenal cannula. The latter was attached after ascertaining that it was not plugged with intestinal contents.

The collected juice was kept chilled at all times and frozen as soon as possible unless used immediately. Clean, sterile glassware and equipment were used at all times. The tube connecting the pancreatic cannula and the collection flask was sterilized in a benzalkonium chloride solution, rinsed with sterile distilled water, and then dried in a sterile test tube at low heat in an oven.

DISCUSSION

The surgical procedure described has proved very satisfactory for its originally designed purpose. The pancreatic secretion obtained met the original requirements in that it was uncontaminated and unactivated, the latter being of prime importance.

We noticed during the development of this technique that the viscosity of the pancreatic secretion varied considerably and, as the viscosity increased, the rate of flow decreased. When the secretion becomes very viscid, there is a tendency toward plugging of the cannula. If the flow from the cannula ceases, it is difficult and at times impossible to re-establish it. It has been our experience that the administration of lactated Ringer's solution intravenously will reduce the viscosity of the pancreatic secretion without materially altering the enzymatic content. In this manner we have been able to avoid serious difficulty when the viscosity of the pancreatic secretion starts to increase to an alarming degree.

Most of the animals prepared in this manner were killed shortly after the desired amount of pancreatic secretion had been collected due to the fact that radioactive isotopes had been used to tag certain carbon materials in the enzymes. However, we did keep some animals for a period of a month, and they showed no functional disorder of the cannula system, nor did they show any gross physiologic change.

Inflammation of the duct did occur in time, followed by a gradual fibrosis of the tissue around the main duct for varying distances, but the degree of inflammation and fibrosis which occurred was not suffi-

cient to eliminate the flow, nor was there any tendency toward activation of the enzymes due to inflammatory exudate.

The protective covering (fig. 5) stayed in place quite well and did not seem to cause any particular problem during maintenance of the animal. The animals were held in stanchions for a while and then in small pens. On some animals it was necessary to resuture the covering after a week or so, but this did not present any large problem.

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The Calcium, Phosphorus, Magnesium, Carotene, and Vitamin A Content of the Blood of Range Cattle in Eastern Montana

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BULLETIN 549 [13] of the Montana Agricultural Experiment Station reports the results of an experimental project designed to correlate soil nutrients, forage nutrients, and nutrient levels of the blood of cattle grazing the forage, with the health, growth rates, and reproductive record of cows on the range at the U.S. Range Livestock Experiment Station at Miles City, Mont. As a part of this project, blood samples for chemical examination were taken from 30 cattle at 28-day intervals for five years, the total number of samples examined being 1,806. As the health and performance of the cattle on this range were satisfactory, it was thought that the blood levels of the constituents determined may be considered as within the normal range for cattle on the northern Great Plains. As published data on the blood chemistry of range cattle are rather limited, it was thought desirable to report the results of the blood analyses separately in a veterinary journal.

MATERIALS AND METHODS

Cattle.—The cattle assigned to the project were 84 yearling Hereford heifers from the large herd of cattle maintained on the U.S. Range Livestock Experiment Station. The number was gradually reduced until there were 60 cows when they had their first calves at the age of 3 years. This number was maintained for three years, until each cow had produced 4 calves. The cattle were divided into six equal lots, one lot in each of six pastures, the pastures being stocked at three grazing intensities.

The cattle were run on grass continuously, with some supplementary feeding in the winter. They were placed in the pastures in May, 1948, when they were approximately 12 months old. During the winter of 1948–1949, they were fed hay at the daily rate of 7 lb. per head from January 24 to March 21, and soybean pellets were fed with the hay from January 14 to March 24. Supplementary hay was fed in the winter of 1949–1950 from December 16 to April 1; in 1950–1951 from January 16 to March 31; in 1951–1952 from December 21 to March 28; and in 1952–1953 from January 7 to April 30. With the exception of the soybean pellets in the first winter, no protein, mineral, or vitamin supplement was fed. In general, the hay fed was of rather poor quality, but in three of the four winters it provided a slightly higher percentage of some nutrients than the feed on the range. It is estimated (Marsh *et al.*[13]) that the over-all average contents of protein, phosphorus, and carotene in the feed actually consumed were 8 per cent protein, 0.12 per cent phosphorus, and 70 μg. per gram of carotene.

The cows were bred during the summer of 1949 when they were 2 years old, and produced four crops of calves during the period of the experiment.

Pastures.—The pastures were located on the native range at the Range Livestock Experiment Station. They consisted of two units, one for summer and one for winter, with each unit being divided into six pastures. These pastures were being used in a long-range study, in which three pairs of pastures in each unit were being continuously stocked at three rates, to compare the effects of heavy, moderate, and light grazing on the range and on the cattle.

The principal grass species in the pastures were blue grama *(Bouteloua gracilis)*, western wheatgrass *(Agropyron smithii)*, spear grass *(Stipa comata)*, and buffalo grass *(Buchloe dactyloides)*. Threadleaf sedge *(Carex filifolia)* was present in considerable quantity.

The principal browse plants were greasewood *(Sarcobatus vermiculatus)*, sagebush *(Artemisia tridentata, A. cana,* and *A. frigida)*, saltbrush *(Atriplex gardneri* and *A. confertifolia)*, and winterfat *(Eurotia lanata)*.

Blood Sampling.—Blood samples were taken every 28 days, from May 20, 1948, to June 11, 1953. Until June, 1950, blood samples were taken from all the animals in heavily and moderately grazed pastures; 28 animals in each of the first seven samplings, 24 in the next eight samplings, then 22 for six samplings, 21 for five samplings, and 19 for the last two samplings before July 1, 1950. From July 13, 1950, to June 11, 1953, blood samples were taken from 30 cows, 5 from each of the six pastures.

Each sample consisted of about 40 ml. of blood drawn into a 50-ml. Rockefeller-type centrifuge tube which contained 1 ml. of a saturated solution of sodium citrate. The blood samples were packed in ice and shipped to the Veterinary Research Laboratory at Bozeman, being delivered at the

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laboratory in the morning following the day of sampling. Immediately after delivery at the laboratory, the samples were centrifuged, and the plasma was drawn off for determination of phosphorus, calcium, magnesium, carotene, and vitamin A.

Analytical Methods.—Phosphorus.—Inorganic phosphorus was determined by a modification of the method of Fiske and Subbarow,[9] in which 2,4-diaminophenol was substituted for 1-amino-2-naphthol-4-sulfonic acid as the reducing agent. This substitution required that the time for color development be increased to 30 minutes because of the less vigorous reducing action of the diaminophenol. Colors developed by this agent are stable for a much longer time than those of the original Fiske-Subbarow procedure.

Calcium.—Readily ionizable calcium was determined by the colorimetric permanganate method of DeLoureiro and Janz[6] after precipitation as the oxalate and washing.

Magnesium.—Readily ionizable magnesium was determined in the filtrate from the calcium determination by precipitating it as magnesium ammonium phosphate by the addition of ammonium hydroxide and ammonium phosphate. The precipitation tubes were rubbed down well to insure complete precipitation, and the precipitate was washed with a mixture of equal volumes of water, alcohol, and ether, containing 1/20 volume of concentrated ammonium hydroxide. The washed precipitate was analyzed for its phosphate content by the phosphorus method described above, and the corresponding magnesium content was calculated.

Carotene and Vitamin A.—These substances were determined by a slight modification of the method of Boyer *et al.*[3]

Results

The plasma levels of phosphorus, calcium, magnesium, carotene, and vitamin A are shown (tables 1, 2). For each constituent, the tables show means, ranges, and standard deviations for each of the 67 samplings. The tables also show the yearly means, the means for the five-year period, and the means for the last three years of the period, during which the cows were producing calves. The yearly figures were calculated from July 1 to June 30, rather than the calendar year. The mean plasma levels for individual cows, during the three-year period when they were producing calves, are shown (table 3) because it is realized that the group averages of tables 2 and 3 do not necessarily give a true picture for individual animals.

Phosphorus.—The highest average of plasma phosphorus for a single sampling of all the cattle tested was 7.7 mg./100 ml., with a range of 5.8 to 8.8 mg./100 ml., on Jan. 27, 1949, when the yearling heifers were receiving a protein concentrate. The highest average when the cattle were grazing natural forage only was 7.6 on May 20, 1948, the first sampling date for the yearling heifers. The lowest average for all cattle tested was 2.1 with a range of 0.7 to 4.4 on April 19, 1951. The annual averages were 5.42 in 1948–1949 and 4.39 in 1949–1950, before the heifers had their first calves. For the following three years, from the time the cows were 3 to 6 years old, the annual averages were 3.5, 3.97, and 3.68. The over-all average for all the cattle for five years was 4.27, and the average for all the cows during the three years of calf production was 3.72.

The annual average phosphorus levels for individual cows for the last three years varied from 2.4 to 5.0, and the three-year averages varied from 2.8 to 4.5, with a range of single samplings of individuals from 0.7 to 9.8. On the three-year average basis there were only 3 cows below 3.4. They were in one of the lots on moderately grazed pasture. They showed no clinical indication of phosphorus deficiency and produced calves each year, but their weight was somewhat lower than that of the other cows in the lot.

Although the general average phosphorus for the three-year period was 3.7, only 12 of the 28 cows had an average of 3.7 or more and 9 of the 12 were in the two overgrazed pastures. The average plasma phosphorus for the other 19 cows was 3.5.

In view of the generally accepted figure of 4.0 mg./100 ml. as the minimum adequate plasma phosphorus, it is of interest that in this series of 1,078 determinations, on samples from 28 cows taken at 28-day intervals over a three-year period, only 418 samples showed a phosphorus level of 4.0 or more milligrams per 100 ml., while 297 had less than 3.0 mg./100 ml. The minimum values for individual cows varied from 0.7 to 2.6, with 22 of the 28 cows showing minimums between 1.0 and 2.0. The maximums varied from 5.0 to 9.8, with 22 of the 28 being between 6.0 and 7.7.

These phosphorus values were in the blood of cows which were giving satisfactory performance in growth and reproduction, except in the two overgrazed lots, which had the highest plasma phosphorus levels.

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TABLE 1—Phosphorus, Calcium, and Magnesium of the Blood Plasma (mg./100 ml.)

Date	No. of cattle	Age of cattle	Phosphorus			Calcium			Magnesium		
			Range	Mean	S.D.	Range	Mean	S.D.	Range	Mean	S.D.
5/20/48	28	1 yr.	6.5–8.9	7.6	0.6	7.3–12.1	10.0	1.1			
6/17			4.6–8.8	6.0	0.8	9.8–12.1	11.2	0.6	1.5–2.2	1.9	0.2
7/15			5.2–7.3	6.1	0.5						
8/12			5.2–10.5	6.4	1.2	10.7–12.6	11.6	0.5	1.4–2.8	2.0	0.3
9/9			2.7–5.2	4.0	0.6	10.0–13.0	11.2	1.2	1.2–2.2	1.8	0.2
10/7			4.1–7.0	5.2	0.7	8.6–13.1	9.7	0.9	1.2–2.7	1.8	0.3
11/4			4.4–6.6	5.3	0.5	9.0–10.8	9.7	0.5	1.6–2.1	1.8	0.2
12/2	24		3.2–5.3	4.3	0.6	8.7–11.1	10.2	0.6	1.1–1.9	1.5	0.2
12/30			2.5–6.8	4.9	1.0	8.8–10.4	9.6	0.5	2.1–3.2	2.6	0.3
1/27/49			5.8–8.8	7.7	0.8	6.9–10.6	8.6	0.8	1.2–3.2	2.2	0.4
2/24			5.3–8.8	6.8	0.7	7.8–10.0	9.0	0.7	0.3–1.9	0.8	0.3
3/24			4.6–8.9	5.7	0.9	9.7–12.9	10.7	1.0	0.0–1.2	0.6	0.2
4/21			4.1–7.3	5.4	0.9	9.0–12.3	10.6	0.7	0.0–2.7	0.6	0.6
5/19			4.0–5.7	4.8	0.4	8.2–11.8	9.0	0.7	0.8–1.4	1.1	0.1
6/16			1.8–8.0	3.8	1.1	10.0–12.2	10.8	0.5	1.3–2.0	1.6	0.2
Year			1.8–10.5	5.42	....	6.9–13.1	10.08	....	0.0–3.2	1.52	....
7/14/49	22	2 yr.	2.2–4.2	3.0	0.5	7.6–10.2	8.9	0.7	1.2–1.7	1.4	0.1
8/11			4.1–6.9	5.4	0.7	10.4–11.7	11.1	0.5	1.2–1.8	1.6	0.2
9/8			2.4–4.5	3.4	0.4	9.1–11.6	10.4	0.6	1.6–2.8	2.3	0.3
10/6			2.3–4.0	3.3	0.5	9.3–12.8	11.0	0.7	1.0–1.9	1.4	0.2
11/3			1.5–4.2	2.9	0.7	9.5–11.0	10.3	0.4	0.8–1.5	1.1	0.2
12/1			2.4–4.8	3.5	0.6	8.6–11.2	10.1	0.6	1.0–1.6	1.2	0.2
12/29	21		3.6–7.2	4.8	1.0	8.0–12.3	10.4	0.9	1.1–1.6	1.4	0.1
1/26/50			2.8–5.9	4.0	0.7	7.7– 9.1	8.2	0.4	0.9–1.5	1.3	0.2
2/23			5.5–9.5	6.6	0.9	9.3–10.9	10.6	0.4	1.2–1.8	1.5	0.2
3/23			3.3–5.4	4.3	0.7	9.8–11.2	10.5	0.3	1.8–2.3	2.1	0.2
4/20			2.0–6.4	3.9	1.2	8.9–10.4	9.8	0.4	0.0–0.4	0.1	0.1
5/18	19		3.8–9.4	6.4	1.8	8.6–11.0	9.3	0.6	0.9–1.7	1.2	0.2
6/15			4.0–7.0	5.6	0.8	10.0–10.0	10.0	0.0	1.6–2.1	1.8	0.1
Year			1.5–9.5	4.39	....	7.6–12.8	10.04	....	0.0–2.8	1.42	....
7/13/50	30	3 yr.	3.3–5.6	4.5	0.6	8.8–11.3	9.7	0.6	1.4–2.1	1.8	0.2
8/10			3.0–5.7	4.3	0.7	8.4–10.2	9.5	0.6	1.9–2.9	2.4	0.3
9/7			2.5–5.4	3.6	0.8	9.8–13.6	11.6	0.8	1.4–2.0	1.7	0.1
10/5			2.3–4.8	3.7	0.7	8.2–14.2	10.8	1.0	1.4–2.3	1.8	0.3
11/2			2.6–4.9	3.6	0.5	9.8–12.7	11.0	0.8	1.2–2.2	1.5	0.2
11/30			2.3–4.4	3.4	0.7	9.6–14.7	11.2	0.7	.....	....	....
12/28			1.7–4.8	3.1	0.6	8.5–11.7	10.3	0.7	0.7–1.7	1.3	0.2
1/25/51			1.4–4.0	2.6	0.7	9.2–12.2	10.4	0.6	1.1–2.0	1.5	0.2
2/22			2.0–4.5	3.0	0.6	9.1–11.2	10.2	0.6	1.6–2.8	2.2	0.2
3/22			1.9–4.5	3.1	0.7	9.7–11.2	10.5	0.4	1.1–2.8	1.8	0.4
4/19			0.7–4.4	2.1	1.0	9.5–11.4	10.6	0.5	1.4–2.2	1.9	0.2
5/17			1.6–5.6	3.3	1.0	9.8–13.3	11.6	0.7	0.7–2.4	1.7	0.6
6/14	29		2.4–9.8	5.2	1.6	9.8–11.4	10.9	0.6	1.0–2.8	1.5	0.4
Year			0.7–9.8	3.50	....	8.2–14.7	10.64	....	0.7–2.9	1.76	....
7/12/51		4 yr.	2.3–6.4	4.5	1.1	8.1–11.6	10.4	0.8	0.9–2.6	1.6	0.4
8/9			1.7–7.4	4.3	1.4	9.0–11.4	10.1	0.7	1.4–2.3	1.8	0.2
9/6			3.7–7.2	5.6	0.8	8.9–10.8	10.0	0.5	1.3–2.8	2.0	0.3
10/4			3.5–7.1	5.0	0.9	7.6–11.9	10.6	1.1	1.3–2.3	1.8	0.3
11/1			3.0–5.5	4.0	0.6	9.0–12.5	10.1	0.7	1.2–1.8	1.5	0.2
11/29			1.0–6.7	3.3	0.9	9.1–10.9	9.3	1.0	1.5–2.1	1.7	0.2
12/27			2.0–5.8	3.7	0.8	8.6–10.3	9.3	0.4	1.2–2.0	1.7	0.2
1/24/52			3.0–5.0	3.8	0.5	8.4–10.4	9.5	0.5	1.2–1.8	1.5	0.1
2/21			2.1–4.9	3.2	0.6	7.8– 9.8	9.0	0.4	1.2–1.8	1.5	0.1
3/20			1.8–3.9	2.8	0.6	9.6–11.2	10.3	0.4	0.9–1.6	1.2	0.2
4/17			1.0–3.8	2.3	0.7	8.0–12.0	9.6	0.9	0.6–1.8	1.2	0.2
5/15			3.4–6.8	5.0	0.9	7.6–10.2	8.6	0.8	0.6–1.7	1.3	0.3
6/12			2.4–6.2	4.2	0.8	8.9–11.1	9.9	0.5	0.9–3.0	1.6	0.3
Year			1.0–7.4	3.97	....	7.6–12.5	9.73	....	0.6–3.0	1.56	....
7/10/52		5 yr.	3.6–6.2	5.1	0.8	8.4–10.3	9.3	0.5	0.7–1.8	1.4	0.2
8/7			2.0–6.8	3.5	1.0	7.7– 9.9	8.7	0.6	1.2–1.9	1.7	0.2
9/4			2.8–6.8	3.9	1.0	8.1– 9.9	9.1	0.5	1.4–2.4	1.7	0.2
10/2			2.2–6.2	3.6	0.8	8.2–10.4	9.3	0.4	1.0–1.8	1.4	0.2
10/30			1.9–5.8	3.7	0.9	7.9– 9.9	9.0	0.5	1.7–2.2	1.9	0.1
11/27			1.4–3.5	2.2	0.5	6.6– 9.8	8.9	0.6	1.0–1.8	1.5	0.2
12/24	28		1.0–3.6	2.2	0.5	7.7–10.2	9.1	0.6	0.9–1.6	1.2	0.2
1/22/53			2.6–5.1	3.9	0.6	7.8–10.0	8.9	0.5	1.2–2.0	1.6	0.2
2/19			3.2–5.8	4.2	0.5	7.2–10.2	9.2	0.5	0.8–1.6	1.3	0.2
3/19			2.4–4.8	3.3	0.6	8.5–10.0	9.1	0.4	0.9–1.4	1.2	0.1
4/16			0.4–4.4	2.2	1.1	8.2–10.0	9.2	0.5	0.7–1.6	1.3	0.2
5/14			2.1–6.8	4.0	1.3	7.0–10.3	9.3	0.7	0.4–2.2	1.0	0.5
6/11		6 yr.	4.2–7.8	6.0	1.1	8.4–10.3	9.4	0.4	1.2–2.8	1.9	0.2
Year			0.4–7.8	3.68	....	6.6–10.4	9.11	....	0.4–2.8	1.46	....
All years			0.4–10.5	4.27	....	6.6–14.7	9.94	....	0.0–3.2	1.55	....
3 years (7/50–6/53)			0.4–9.8	3.72	....	6.6–14.7	9.8	....	0.4–3.0	1.59	....

TABLE 2—Carotene and Vitamin A of the Blood Plasma (μg./100 ml.)

Date	No. of cattle	Age of cattle	Carotene			Vitamin A		
			Range	Mean	S.D.	Range	Mean	S.D.
5/20/48	28	1 yr.	235–660	442	95	22–43	36	5
6/17			200–530	350	83	24–34	28	3
7/15			155–457	288	82	18–33	27	4
8/12			168–436	283	78	23–45	32	5
9/9			100–410	260	88	24–38	32	3
10/7			50–225	125	49	22–41	33	4
11/4			0–96	46	27	20–41	31	5
12/2	24		5–48	24	13	23–38	30	4
12/30			2–35	10	6	.....	....	..
1/27/49			0–16	4	3	14–29	20	4
2/24			10–40	22	7	11–22	18	3
3/24			5–10	9	2	11–27	19	4
4/21			290–650	434	81	26–37	33	3
5/19			340–760	569	102	20–39	27	5
6/16			300–650	476	101	15–44	30	6
Year			0–760	223	.....	11–45	28	..
7/14/49	22	2 yr.	100–260	194	43	19–38	31	5
8/11			65–130	92	20	16–30	22	4
9/8			55–158	115	26	26–44	36	5
10/6			40–140	95	27	20–39	30	5
11/3			70–210	122	32	28–47	41	5
12/1			95–300	179	62	22–36	29	4
12/29	21		80–180	113	25	21–36	28	4
1/26/50			30–85	48	16	14–30	23	4
2/23			30–95	61	14	20–44	32	7
3/23			50–120	75	19	31–47	38	6
4/20			30–410	205	97	12–34	24	6
5/18	19		500–800	652	95	4–33	26	7
6/15			350–730	551	131	23–42	33	5
Year			30–800	192	.....	4–47	30	..
7/13/50	30	3 yr.	350–870	621	133	29–44	33	3
8/10			195–680	440	107	17–54	30	8
9/7			105–365	197	98	22–53	39	7
10/5			47–386	181	61	32–45	38	4
11/2			110–270	194	44	21–40	31	5
11/30			40–150	72	25	31–47	40	4
12/28			16–70	47	11	19–48	35	6
1/25/51			25–60	42	9	23–41	33	4
2/22			20–55	38	9	18–31	26	4
3/22			20–48	31	6	20–33	26	4
4/19			224–465	335	67	22–47	35	5
5/17			386–960	657	133	9–30	20	4
6/14	29		300–760	514	113	28–49	39	5
Year			20–960	259	.....	9–54	33	..
7/12/51		4 yr.	450–1,000	728	147	22–50	36	6
8/9			145–830	387	140	16–41	30	6
9/6			180–610	322	92	14–39	30	6
10/4			512–913	680	124	.....	....	..
11/1			195–465	317	74	28–44	35	5
11/29			60–230	142	41	30–44	38	4
12/27			24–60	43	13	16–35	27	4
1/24/52			13–56	29	10	9–42	26	8
2/21			9–45	26	8	17–39	28	5
3/20			32–95	55	16	16–29	21	4
4/17			78–215	138	39	9–34	21	6
5/15			247–1,000	487	149	19–42	31	6
6/12			340–800	508	111	24–40	32	5
Year			9–1,000	299	.....	9–50	29	..
7/10/52		5 yr.	270–730	447	112	7–46	27	10
8/7			162–465	263	75	19–36	26	5
9/4			81–320	148	51	18–34	25	4
10/2/52			17–111	56	21	15–36	29	6
10/30			12–36	22	7	25–39	32	4
11/27			9–32	17	6	15–41	32	5
12/24	28		13–32	21	5	22–38	29	4
1/22/53			52–132	81	19	15–37	25	4
2/19			45–120	77	19	16–36	25	4
3/19			56–163	102	25	22–36	29	4
4/16			88–195	141	28	13–36	26	6
5/14			589–1,000	853	132	20–42	32	5
6/11		6 yr.	570–1,100	902	167	28–50	37	7
Year			9–1,100	237	.....	7–50	29	..
All years			0–1,100	242	.....	4–54	29	..
3 years (7/13/50–6/11/53)			9–1,100	265	.....	7–54	30	..

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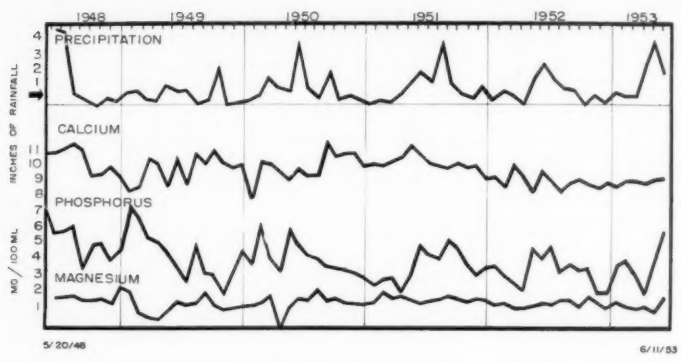

Fig. 1—Graphs showing monthly precipitation from May, 1948, to June, 1953, and the mean plasma levels of phosphorus, calcium, and magnesium of cattle on pasture, determined at 28-day intervals.

The seasonal variation in plasma phosphorus on the basis of means of all cattle tested is shown (fig. 1). In general, the level is high in May, June, and July, dropping in the late summer and fall to winter lows. This general trend was broken in the winter of 1948–1949, because of feeding of concentrate, which produced a high point in January, 1949. Low rainfall in 1949 prevented the usual rise during the growing season. Again, during the winter of 1949–1950, hay feeding reduced the winter drop in phosphorus.

The graph (fig. 1) also shows that the plasma phosphorus curve roughly parallels the precipitation curve, although this relation was somewhat modified by supplementary winter feeding.

Calcium.—The average of plasma calcium for single samplings of all the cattle tested ranged from a high of 11.6 mg./100 ml. on three dates to a low of 8.2 mg./100 ml. on Jan. 26, 1950. The annual means were 10.08, 10.04, 10.64, 9.73, and 9.11, with an over-all average of 9.94. The significance of the drop in plasma calcium during the last two years is not known.

The annual average calcium levels for each of the cows for the last three years varied from 8.4 to 11.5, and the three-year averages varied from 9.1 to 10.5. The minimum and maximum values for single samplings of individuals were 6.6 and 14.7.

The graphs in figure 1 do not show any consistent pattern of seasonal variation in plasma calcium, nor relation to the phosphorus content of the plasma.

Magnesium.—The average magnesium levels for single samplings of all the cattle ranged from a high of 2.6 mg./100 ml. on Dec. 30, 1948, to a low of 0.1 mg./100 ml. on April 20, 1950. The levels in individual cows varied from 0.0 to 3.2. The annual means were quite constant, ranging from 1.42 to 1.76. The over-all average for the five years was 1.55 mg./100 ml.

There were no significant differences between individual cows in the annual and three-year averages of plasma magnesium levels. The annual averages varied from 1.1 to 2.1, and the three-year averages varied from 1.2 to 1.9. The minimum and maximum values for single samplings during the last three years were 0.4 and 2.9.

In each of the five years, the low point occurred in one of the spring months. In 1949 and 1950 there was a marked drop in April. In the following three years, the low point for the year occurred in April, May, or June, but in these years these low points were only slightly lower than the average for the year. The low magnesium levels occurred at the time of the year when the hypomagnesemic disease known as "grass tetany" characteristically occurs. This condition was not observed in the experimental cattle, but veterinarians report that it is not uncommon in the area.

Carotene.—As would be expected, the plasma carotene showed a wide range, the means for all the cattle varying from a high of 900 μg./100 ml. in June, 1953, to a low of 4 μg./100 ml. in January, 1949. The annual means did not show a wide range, varying from 192 to 299, with an over-all average of 242.

There was no significant difference between individual cows in the carotene content of the blood. The annual individual averages for the last three years varied from 180 to 426 μg./100 ml., and the three-year averages varied from 197 to 375. The

minimums for the individual cows varied from 9 to 25, while the maximums varied from 589 to 1,100. Of 1,079 determinations on 28 cows over a period of three years, 649 showed a carotene level higher than 100 μg./100 ml.

The peak for each year occurred in May, June, or July, followed by a rapid decline, with very low values in midwinter (fig. 2). The summer of 1951 was an exception. In that season, there were three peaks, one in May, one in July, and one in October. The October peak followed unusual September rainfall and growth of green feed.

Vitamin A.—The average plasma vitamin A for each sample from all the cattle varied from a high of 41 μg./100 ml. to a low of 18 μg./100 ml., with a range for single samples of 4 to 54. The annual means varied from 28 to 33 with an over-all mean of 29 μg./100 ml.

Out of approximately 1,800 individual samples, only 145 were below 20 μg./100 ml. Of these, 43 were on the three dates in the winter of 1949, while the remaining 102 were scattered through the entire period.

The annual average vitamin A levels of the individual cows for the last three years varied from 23 to 40 μg./100 ml., and the three-year averages varied from 26 to 36. The individual minimum and maximum was 7 and 53. Of 1,038 samples taken at 28-day intervals over a period of three years, 124 were below the 22 μg./100 ml. which is stated by Davis and Madsen [5] to be the minimum adequate for a breeding cow. The samples that were below 22 were distributed among 26 of the 28 cows.

The seasonal variation in the average vitamin A values did not follow the carotene variations closely, although the only three dates (January, February, and March, 1949) when the average vitamin A was below 20 μg./100 ml. corresponded to the lowest carotene values for the entire period.

Discussion

In reporting the results of the analyses for phosphorus, calcium, magnesium, carotene, and vitamin A in the blood plasma of these cattle, it is believed that we may be presenting data which can be considered as establishing normals for range cattle in the northern Great Plains area. This opinion is based on published information [13] which shows, first, that the five-year period during which the blood samples were taken was normal for eastern Montana in precipitation; and, second, that the performance of the cattle, except in the overgrazed pastures, was satisfactory as to health, growth, and reproductive efficiency.

The annual precipitation at Miles City for the six calendar years involved in the experimental period was 15.3 inches in 1948, 8.8 in 1949, 13.7 in 1950, 14.9 in 1951, 9.9 in 1952, and 16.6 in 1953. The average was 13.2 inches, which corresponds with the 13.18 inches shown by the weather bureau as the average for 78 years at Miles City. The experimental period included

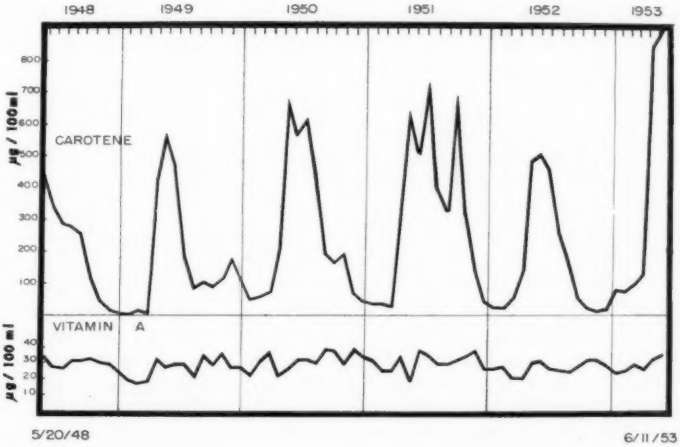

Fig. 2—Graphs showing the mean plasma levels of carotene and vitamin A of cattle on pasture, determined at 28-day intervals from May 20, 1948, to June 11, 1953.

two very dry seasons, two with above average moisture, one average season, and parts of two years with moisture above average.

Where the pastures were not overstocked, there was no evidence of nutritional deficiency either clinically or as measured by the weights of the cows and their reproductive efficiency. In the fall of 1952, when the cows were 5 years old, the average weight of the cows nursing calves was 1,161 lb. in the lightly grazed pastures and 1,112 lb. in the moderately grazed pastures. The average calving percentage of the cows in the lightly and moderately grazed pastures was 92 per cent, with calf weaning weights of 439 lb. and 422 lb. While the cattle in the overgrazed pastures showed no evidence of specific deficiencies, the average weight of the wet cows in the fall of 1952 was only 1,039 lb. and the average calving percentage was 77 per cent, with calf weaning weight of 372 lb.

As previously indicated, one set of pastures was overgrazed, one was moderately grazed, and one set was lightly grazed. Although the cattle in the overgrazed pastures showed the effects in lower weights and lower calf crop, these effects were not reflected in the levels of the blood plasma constituents. There were no significant differences in the plasma levels of calcium, magnesium, carotene, and vitamin A among cattle at the three grazing intensities.

The results of the comparison of phosphorus levels of cows in the three groups were interesting and unexpected. As no blood samples were taken from the animals in the lightly grazed pastures during the first two years, the comparisons are made for the last three years when the cows were producing calves. The averages of the means for the three years were 4.17 mg./100 ml. for the heavy grazing intensity lots, 3.38 mg./100 ml. for the moderate grazing intensity, and 3.64 mg./100 ml. for the cattle in the lightly grazed pastures. The same relative positions of the three groups existed for each of the three years. Furthermore, the higher plasma phosphorus level in the cattle on the overgrazed pastures occurred in nearly all the 39 samplings. In 37 of the 39 samplings, the plasma phosphorus was greater in the overgrazed lot than in the lot in the moderately grazed pastures, and in 32 samplings it was greater than in the cattle at the light grazing intensity. The reason for this situation is not clear. It was suggested that the fact of lower calf production in the overgrazed pastures might explain the higher blood phosphorus but, when the dry cows were eliminated from the calculations, the phosphorus levels were still consistently higher in the cows on the heavily grazed pastures. Another condition which may be a factor in this situation is the lack of old feed of a low phosphorus content in the heavily grazed pastures, so that the cattle were more constantly limited to growing plants carrying a higher percentage of phosphorus. While the differences in calcium levels between the three grazing intensities were not significant, they are reciprocal to the phosphorus differences. The three-year average for the heavy grazing intensity lots was 9.55 mg./100 ml., for the moderate grazing lots 10.1 mg./100 ml., and for the lightly grazed lots it was 9.85 mg./100 ml.

The results of the determination of the plasma levels of inorganic phosphorus, calcium, magnesium, carotene, and vitamin A are summarized (table 4) to show the averages of the monthly means for the five-year period covering ages from 1 to 6 years; for yearlings, 2-year-olds, and for breeding cows from 3 to 6 years old. For breeding cows, the average values for 39 samplings over a three-year period were: for phosphorus, 3.7 mg./100 ml.; calcium, 9.8 mg./100 ml.; magnesium, 1.6 mg./100 ml.; carotene, 265 μg./100 ml.; vitamin A, 30 μg./100 ml. The over-all averages were not significantly different from those for the breeding cows except in the case of phosphorus, which was 4.3 mg./100 ml. The yearling heifers averaged 5.4 mg./100 ml., and the 2-year-old heifers averaged 4.4 mg./100 mg.

Comparing the values for breeding cows (beef) with values found in the literature, which may be interpreted as normal for beef cows, we find that our three-year average for phosphorus (3.7 mg./100 ml.) is slightly below the figure which has usually been considered a normal average. Dukes[7] gives the normal range for cattle as 3.0 to 8.0 mg./100 ml., but does not suggest an annual average. DuToit *et al.*[8] give 3.2 mg./100 ml. in whole blood as normal for lactating cows in South Africa, which corresponds to 4.1 mg./100 ml. in plasma. Stanley[19] reported average plasma phosphorus in range cows in Arizona of 4.97 mg./100

ml. during one year and 5.37 mg./100 ml. during another year. Josland [11] in New Zealand gave a general average for lactating cows of 4.3 mg./100 ml. Wheeler,[23] in Wyoming, said that "For mature cattle normal values range between 4.0 and 5.5 milligrams per cent." Johnson *et al.*,[10] in South Dakota, found that three-year averages for samplings on December 1, March 1, and August 1 were 4.64, 4.54, and 5.40 mg./100 ml., respectively. These levels were considered as normal but near the low limit of adequacy.

Watkins and Knox [21] reported that range cows in New Mexico with average plasma phosphorus levels varying from 2.11 mg./100 ml. during the winter to 5.37 mg./100 ml. during the summer, and with an over-all average of 3.53 mg./100 ml., showed excellent growth and production rates. Nelson *et al.*[14] reported satisfactory performance from a group of cows in Oklahoma with an over-all average of 4.3 mg./100 ml., while another lot with an over-all average of 3.2 mg./100 ml. was below par in performance. Reynolds *et al.*[17] found that range cows with an over-all average of 3.05 mg./100 ml. of whole blood were below par in growth and reproduction, while cows with an average of 4.3 mg./100 ml. of whole blood performed satisfactorily. These values would correspond to about 4.0 and 5.6 mg./100 ml. of plasma. Payne *et al.*[15] found an average plasma phosphorus of 4.89 mg./100 ml. for adult Hereford cows on grass in Wyoming, the samples being taken at the end of lactation and in the early stages of gestation. Davis *et al.*[4] report an average phosphorus level in Florida beef cattle of 4.6 mg./100 ml., with no seasonal variation.

Our findings confirm those of Watkins and Knox [21] in showing that an average plasma phosphorus level between 3.5 and 4.0 mg./100 ml. in range cows indicates adequate phosphorus nutrition though winter values may be much lower. The reports of Nelson [14] and Reynolds *et al.*[17] indicate that our over-all average of 3.7 mg./100 ml. is near the minimum adequate.

Our three-year average for plasma calcium in breeding cows of 9.8 mg./100 ml. is within the normal range given by Dukes [7] of 9.0 to 12.0 mg./100 ml.. Wheeler [23] found a somewhat higher average of 11.7 mg./100 ml. in range cows in Wyoming. Johnson *et al.*[10] found an average of 9.7 mg./100 ml. in range cows in South Dakota, which was considered normal. Stanley [19] reported 10.25 and 10.86 mg./100 ml. in two series

TABLE 3—Phosphorus, Calcium, Magnesium, Carotene, and Vitamin A in the Plasma of Individual Breeding Cows, Showing Means and Ranges for the Three Year Period July 13, 1950, to June 11, 1953

		Phosphorus		Calcium		Magnesium		Carotene		Vitamin A	
Lot	Cow	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
	1	4.4	1.7–9.8	9.6	7.0–12.9	1.6	0.9–2.7	233	15–912	29	17–42
	10	4.1	1.7–6.6	9 5	7.6–11.7	1.5	0.9–2.8	197	13–730	29	17–44
	12	3.8	1.4–6.6	9.9	8.4–11.7	1.6	1.0–2.5	252	15–880	28	14–41
Overgrazed	13	4.2	1.6–6.8	9.1	7.2–10.9	1.6	1.0–2.5	256	20–880	26	7–36
pastures	58	4.5	2.6–7.4	9.2	8.0–11.5	1.4	0.4–2.3	281	13–910	31	12–47
	59	3.9	2.2–7.6	9.5	7.7–11.5	1.6	0.7–2.5	233	9–1,070	29	9–42
	62	3.8	2.0–5.0	9.7	7.7–11.8	1.8	1.2–2.4	375	20–1,100	36	9–50
	64	3.9	1.7–6.8	10.0	8.4–12.4	1.5	0.8–2.6	208	13–589	31	9–42
	67	4.4	2.4–6.6	9.8	7.8–12.5	1.6	0.7–2.8	271	15–870	30	12–42
	29	2.9	0.7–6.1	10.2	6.6–13.3	1.5	1.0–2.4	318	15–1,100	33	16–44
	30	2.8	0.8–5.6	10.1	8.8–14.2	1.6	0.8–2.8	230	13–1,010	29	16–41
	32	3.6	1.7–6.4	9.9	8.0–11.8	1.6	0.6–2.6	299	20–1,010	33	19–48
	33	2.9	1.0–6.2	10.4	8.8–13.3	1.6	0.9–2.6	295	20–1,100	35	19–50
Moderately	40	3.6	1.5–6.4	10.1	8.5–12.7	1.5	0.4–2.7	305	20–980	34	20–47
grazed	44	3.4	1.3–6.2	9.8	8.4–12.7	1.5	0.8–2.5	293	13–1,100	31	20–45
	45	3.6	1.7–7.7	10.2	7.8–12.0	1.2	0.4–2.5	255	12–870	30	17–49
	46	3.5	1.0–7.6	9.8	8.6–11.8	1.8	1.0–2.7	283	15–960	30	16–44
	47	3.8	1.0–7.3	10.5	8.2–14.7	1.7	1.0–2.8	271	16–1,000	30	16–48
	49	3.5	0.7–7.8	10.1	8.5–11.9	1.7	1.1–2.8	353	25–1,100	29	12–43
	16	4.0	1.2–6.5	9.2	7.2–11.8	1.7	0.8–2.4	241	15–762	26	16–37
	17	3.5	1.0–7.2	9.8	8.0–12.4	1.9	1.2–2.6	291	13–1,070	31	15–44
	24	3.6	1.2–5.5	9.7	8.4–12.2	1.5	0.6–2.9	203	12–760	28	13–48
Lightly	26	3.5	1.0–6.0	10.3	8.8–13.6	1.7	1.1–2.8	267	13–870	27	15–41
grazed	28	3.4	1.4–6.9	10.1	7.7–12.6	1.7	0.9–2.5	284	20–1,070	29	18–53
	72	3.6	1.4–5.5	9.9	8.5–11.7	1.5	0.5–2.5	238	15–870	30	16–41
	77	3.7	1.0–6.8	10.0	7.7–12.2	1.7	0.8–2.3	286	13–1,100	31	12–44
	80	3.4	1.8–5.5	9.7	7.7–11.3	1.5	0.6–2.2	249	9–910	29	15–44
	81	3.6	1.5–6.5	9.9	8.4–11.4	1.5	0.7–2.3	221	13–800	34	11–47

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TABLE 4—Averages of Monthly Means of Plasma Levels of Phosphorus, Calcium, Magnesium, Carotene and Vitamin A, with Minimum and Maximum Means and Ranges for Individual Cows

	All cattle	Yearlings	2-yr.-old	Breeding cows—7/50–6/53			
	5/48–6/53	5/48–6/49	7/49–6/50	3-yr. av.	Low monthly mean	High monthly mean	Range for individual cows
Phosphorus	4.3	5.4	4.4	3.7	2.1	6.0	0.4– 9.8
Calcium	9.9	10.1	10.0	9.8	8.6	11.6	6.6–14.7
Magnesium	1.5	1.5	1.4	1.6	1.0	2.4	0.4– 3.0
Carotene	242	223	192	265	17	902	9–1,100
Vitamin A	29	28	30	30	20	39	7–54

of samples from Arizona range cows. Payne *et al.*[15] found an average of 9.52 mg./100 ml. in cows in Wyoming. Josland[11] reported an average plasma calcium level of 9.5 mg./100 ml. for lactating cows in New Zealand. Robinson and Huffman[18] found an average in 122 beef cattle of 11.0 mg./100 ml. Davis *et al.*[4] reported a calcium average of 10.8 mg./100 ml. in Florida beef cattle.

The general average magnesium level (1.6 mg./100 ml.) in the plasma of the breeding cows in our experiment appears to be slightly low. We have been unable to find in the literature an accepted normal range of magnesium level in beef cows, but it appears that, in general, a magnesium level below 2.0 mg./100 ml. is considered below normal. Stewart and Reith[20] stated that the normal level for cows is 2.5 to 3.0 mg./100 ml., and that 2.0 mg./100 ml. is the lower limit of the normal range. Blaxter and Rook[2] used 2.15 mg./100 ml. as the criterion of normality in calves. Allcroft and Green[1] found a normal range in dairy cows of 1.85 to 3.17 mg./100 ml.

The three-year average for carotene of 265 μg./100 ml. is probably of little significance except as indicating that the feed consumed supplied more than sufficient carotene. Watkins and Knox[22] reported monthly carotene determinations for a five-year period on the plasma of range cows in New Mexico. The five-year average for each month of the year varied from 163 μg./100 ml. for January to 727 μg./100 ml. for June, with an over-all average of 382 μg./100 ml. Long *et al.*[12] reported averages in range cows in Oklahoma varying from 71 μg./100 ml. in February to 1,027 μg./100 ml. in June. Johnson *et al.*[10] reported mean values for range cows from 59 μg./100 ml. in December to 411 μg./100 ml. in August.

The three-year vitamin A average for the breeding cows, 30.0 μg./100 ml., is well above the critical level of 22.0 μg./100 ml. determined by Davis and Madsen.[5] Johnson *et al.*[10] reported means of 23.6 μg./100 ml. in December and 61.0 μg./100 ml. in August, with individual minimums of 3.0 μg. in December and 13.0 μg. in March. Long *et al.*[12] found means ranging from 9.3 μg./100 ml. in April to 47.4 μg./100 ml. in August. Watkins and Knox[22] reported five-year averages for each month of the year ranging from 44.0 μg. in January to 60.0 μg. in June. Repp and Watkins[16] found average plasma vitamin A levels in cows on six New Mexico ranches ranged from 20.0 μg./100 ml. to 45 μg./100 ml., with individuals as low as 13.0 μg./100 ml.

SUMMARY

The phosphorus, calcium, magnesium, carotene, and vitamin A content of the blood plasma of Hereford cattle at the U.S. Range Livestock Experiment Station at Miles City, Mont., was determined at 28-day intervals over a period of five years.

Since these cows performed satisfactorily as measured by general health, growth, and reproduction, when the range was not overstocked, the values obtained may be considered normal and adequate for range cattle in much of the northern Great Plains area.

For breeding cows, the average values for 39 samplings during a three-year period were: phosphorus, 3.7 mg./100 ml.; calcium, 9.8 mg./100 ml.; magnesium, 1.6 mg./100 ml.; carotene, 265 μg./100 ml.; and vitamin A, 30 μg./100 ml. The values for these same cattle when they were yearlings and 2-year-olds did not differ significantly except in the case of phosphorus, which was higher in the heifers than in the breeding cows.

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Electrophoretic Studies of Bovine Serum. III. Serum Protein Changes in Anaplasmosis

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ALTHOUGH MANY REPORTS on bovine anaplasmosis have been made since it was first described approximately 60 years ago,[2] the majority of the information has been clinical. In recent years, studies have been directed toward definition of the etiologic agent,[8-10, 19] diagnosis,[11, 12] transmission,[3, 14] and pathologic changes,[17] but basic knowledge concerning the physicochemical alterations produced in the host as a result of *Anaplasma marginale* infection is still lacking. In one report,[16] three serum samples from an animal infected with *A. marginale* were analyzed for various biologic components; however, details on the substances and methods of analysis were scanty and undefined.

For these reasons, it became of interest to conduct a study of serum proteins of cattle suffering from anaplasmosis. The present report gives data obtained in electrophoretic analyses of serum proteins from calves which were experimentally infected with *A. marginale*. The serum components that were of most interest to study were the total serum proteins (TSP), total globulins (TG), and individual globulins. Studies of these proteins in other pathologic conditions have shown that the changes observed are related to the progress of the diseases and the formation of antibodies.[1, 4, 13, 15]

MATERIALS AND METHODS

A total of 16 splenectomized dairy calves of mixed breeds, 2 to 6 months old, were used in this phase of the study. Details concerning calf management procedures, methods of preparation of blood smears for the detection of infections of anaplasmosis and eperythrozoonosis, and protein analyses using paper electrophoretic and protein nitrogen techniques have been described in reports on postsplenectomy changes in the serum proteins[5] and on hypoglobulinemia produced in eperythrozoonosis.[6]

In order to produce experimental infections of anaplasmosis, whole citrated blood was used as the inoculum. It was obtained from carrier animals, those that had recovered from the disease, or from calves which showed Anaplasma bodies (AB) in a relatively large number of their erythrocytes. The experimental calves were given 10 to 250 ml. of infective blood by the intravenous route.

RESULTS

The values of the TSP, TG, and individual globulins are given in absolute figures; *i.e.*, in grams per 100 ml. of serum. The albumin/globulin (A/G) ratio, which denotes the proportion of albumin to globulin, is given as a relative value and does not indicate a quantitative figure. The TSP and A/G ratio give absolute and relative information, respectively, on the entire serum protein picture, whereas TG and individual globulin values present absolute information on these fractions. The globulin fractions contain antibodies[7] and are probably responsible for protection and immunity. The globulins may also be responsible for the serologic activity[4, 13] such as may be found using the complement-fixation test for anaplasmosis.[11, 12, 18]

Results presented here are for changes observed after recovery from surgery when the serum proteins had returned to preoperative levels[5] and after inoculation with infective blood.

The average period of time that AB became evident in these calves was approximately 11 days after inoculation with infective blood. Approximately six days prior to microscopic evidence of AB in the peripheral blood and five days after inoculation, the concentrations of TSP and all globulins decreased. A slight decrease in the value of the A/G ratio was also observed. The concentrations of all globulins and TSP decreased further until one day prior to the appearance of AB, whereas the A/G ratio increased during this interval and until

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the thirteenth day after inoculation. Simultaneous with the appearance of AB on the eleventh day after inoculation, increases were noticed in the concentrations of TSP until the seventeenth day postinoculation, at a time when the AB count had reached its highest level. *Alpha-* and *beta*-globulin concentrations increased during the eleventh to the seventeenth day after inoculation, although *gamma*-globulin levels did not show appreciable changes. The TG concentrations reached a high level on the sixteenth day after inoculation, just one day before a high AB count was reached, and remained essentially static until the end of the observation period at 62 days.

The concentration of TSP showed a second increase on the nineteenth day after inoculation and persisted for an additional five days as the number of AB began to decrease. After this interval, the concentrations of TSP steadily increased until the end of the experimental period.

Alpha-globulin concentrations began to decrease after the seventeenth day postinoculation and returned to pre-exposure levels by the 27th day. They remained at this level until the study was terminated. *Beta*-globulin levels also decreased during this interval and returned to preinoculation values by the 34th day after inoculation.

Gamma-globulin concentrations increased progressively from the nineteenth day after inoculation until the end of the observation period; they reached higher levels than preinoculation concentrations by the 34th day after inoculation. The elevated concentrations of *gamma*-globulin remained until the end of the observation period.

A second decrease in the A/G ratio was observed from the fourteenth day until the 24th day after inoculation. At this time, A/G ratio values steadily increased and returned to pre-exposure values by the time the study was terminated.

Mean serum protein values and AB percentages are given (table 1) for the 16 calves. All values were calculated as means after peak AB counts for each calf were superimposed. The figures given are means for each serum value and AB percentage at

TABLE 1—Electrophoretic Analyses of Serums from 16 Dairy Calves Experimentally Infected with Anaplasmosis

Days (blood sampling intervals)	Mean serum protein values						Anaplasma bodies (%)‡
			Globulins†				
	TSP*	A/G**	*Alpha*	*Beta*	*Gamma*	Total	
−1	6.65	0.81	1.12	1.14	1.42	3.68	0
0			INOCULATED				0
1	6.71	0.82	1.14	1.17	1.38	3.69	0
3	6.80	0.82	1.18	1.15	1.40	3.73	0
5	6.10	0.76	1.06	1.11	1.29	3.46	0
8	5.52	0.94	0.82	0.89	1.13	2.84	0
9	5.34	0.98	0.87	0.90	0.92	2.69	0
10	5.38	1.09	0.86	0.83	0.88	2.57	0
11	5.47	0.90	0.94	0.99	0.95	2.88	<1.0
12	5.63	0.97	0.98	0.97	0.91	2.86	3.2
13	5.76	0.97	1.07	0.96	0.89	2.92	7.4
14	5.86	0.82	1.12	1.17	0.93	3.22	13.6
15	6.22	0.80	1.20	1.28	0.96	3.44	29.3
16	6.51	0.67	1.49	1.54	0.88	3.91	31.5
17	6.93	0.73	1.57	1.60	0.84	4.01	42.7‖
19	6.44	0.64	1.52	1.48	0.93	3.93	32.7§
20	6.26	0.58	1.39	1.40	1.17	3.96	35.2
21	5.92	0.48	1.44	1.48	1.09	4.01	30.7
22	5.53	0.41	1.40	1.39	1.14	3.93	26.4
24	5.12	0.29	1.28	1.49	1.20	3.97	24.8
26	6.30	0.58	1.26	1.46	1.28	4.00	14.5
27	6.45	0.60	1.13	1.54	1.36	4.03	5.8
28	6.62	0.66	1.10	1.51	1.37	3.98	4.0
29	6.79	0.71	1.08	1.48	1.40	3.96	4.5
32	6.81	0.73	1.09	1.30	1.54	3.93	1.4
33	6.84	0.76	1.05	1.25	1.58	3.88	1.2
34	6.88	0.74	1.06	1.17	1.73	3.96	<1.0
39	6.97	0.78	1.05	1.04	1.82	3.91	<1.0
47	7.04	0.71	1.11	1.12	1.88	4.11	0
54	7.02	0.72	1.06	1.06	1.89	4.01	0
62	7.09	0.79	1.04	1.05	1.88	3.97	0

* TSP = total serum proteins as grams per 100 ml. of serum. ** A/G = albumin/globulin ratio. † Globulins = grams per 100 ml. of serum. ‡ Anaplasma bodies = percentage of erythrocytes containing Anaplasma bodies. ‖ Two calves died. § One calf died.

XUM

the various stages during the course of the experimental infection.

The results of serum protein analyses of normal calves that were splenectomized and had recovered from surgery have been given in a previous report.[5]

DISCUSSION

It is generally known that the time of appearance of AB in the peripheral blood is variable before clinical signs of the disease become evident in infected cattle.[2] Observations made in the present study show that a debilitating effect occurs in the serum proteins approximately six days before the appearance of AB in the erythrocytes. All serum proteins decrease in concentration. The proportion of albumin to globulin, as shown by the A/G ratio, also decreases slightly in value. However, these decreases cannot be applied as diagnostic criteria for anaplasmosis; as in most diseases, not all of the changes observed in the serum proteins are specific and indicative of one particular disease. (Two diseases in which these changes are indicative are multiple myeloma,[20] where there is an abnormal serum protein, and eperythrozoonosis, where hypoglobulinemia is produced [6].) The deviations from the normal only give indications of the current status of the serum proteins of the diseased animal.

It is likely that the rise in TSP at approximately 15 days after inoculation was a direct result of infection with *A. marginale*, since it was at this time that *alpha-* and *beta*-globulins increased. These increases were concurrently reflected as an increase in the TG at this time with no changes in the concentration of *gamma*-globulin. During the second increase in TSP at approximately 26 days after inoculation, *gamma*-globulin had begun to increase in concentration while the levels of *alpha-* and *beta*-globulins were returning to pre-exposure levels. Increases in TSP and globulins have been found to parallel antibody titers in many infectious conditions, particularly those of bacterial origin.[1,15] Possibly antibodies are resident in the *alpha-* and *beta*-globulins early in the course of the disease but appear in the *gamma*-globulin as the disease runs its course. Complement-fixing antibodies have been shown to appear approximately one to two weeks after inoculation with infective blood, and titers are then maintained for months at relatively high levels.[11,12,18] Studies are in progress to determine if a relationship exists between antibody titers and weights of various serum protein components and to determine the residence of antibodies in the serum components during different stages of anaplasmosis. It has been shown that different antibodies may be present in different serum fractions in various diseases.[4,7,13]

The second decrease in TSP at approximately 19 days after inoculation was maintained for about seven days and may be directly due to an inhibition in the production of serum proteins, in this case in the production of albumin by the liver, which has been shown to be a site of albumin synthesis.[13] This decrease in TSP and the subsequent decrease in the concentration of albumin was reflected by the depressed A/G ratio during this time. It has been established that pathologic changes occur in the liver in anaplasmosis.[17] Possibly the liver may produce less albumin because of these pathologic changes. Globulin levels remained elevated during this time, as indicated by the decrease in the A/G ratio.

The work of Piettre,[16] in which two samples of serum obtained from an animal during the incubation and febrile stages of anaplasmosis infection were analyzed, showed no appreciable changes in what were termed "dry extract," "serum globulin," "myxoprotein," "albumin," "lipids," and "chloride." During the convalescent period, Piettre observed increases in the amounts of dry extract and myxoprotein but a decrease in the serum globulin. Since definite times in the course of the disease and methods of analyses were not given, adequate comparisons cannot be made with the present data. If TSP can be likened to dry extract, then the increases compare favorably during convalescence. If TG can be compared with the serum globulin of Piettre, then there is lack of agreement in the changes observed during the convalescent period.

SUMMARY

1) Changes in the serum proteins of calves infected with *Anaplasma marginale* were studied.

2) Prior to the appearance of Anaplasma bodies there were decreases in the concentrations of all serum proteins.

3) A peak in the Anaplasma body count was paralleled by increases in the total serum proteins, *alpha-* and *beta*-globulins, and total globulins. The concentration of *gamma*-globulin was low at this time.

4) As the number of Anaplasma bodies decreased, the albumin/globulin ratio decreased to its lowest value during the observation period, as did the concentration of total serum proteins.

5) An increase in the concentration of *gamma*-globulin occurred as the level of *alpha-* and *beta*-globulins returned to pre-infection values.

6) Various aspects concerning these changes are discussed.

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A Preliminary Report on the Serum Protein-Bound Iodine in Dairy Cattle

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DURING the past three years, workers in the Department of Animal Disease Research at the Alabama Polytechnic Institute have studied the cause of infertility or "repeat breeding" in cattle. Investigations have been conducted on the bacterial flora of cervical mucus in relation to breeding. A large percentage of cultures made from cows requiring more than three services have been negative and it has been impossible to associate any specific pathogenic infection or infections with the general problem of infertility. Therefore, it is postulated that some physiologic or hormonal deficiency or dysfunction may be an important etiologic factor.

With confirmation in mind, a preliminary step was to determine the relative activity of the thyroid gland, using serum protein-bound iodine (PBI) as an index in a fairly large group of animals picked at random from the various herds in which sterility work was being done. It was desired, in an initial investigation, to determine the normal range and the average PBI values in dairy cattle and to determine whether infection or abnormal circumstances would substantially change these values.

REVIEW OF LITERATURE

The importance of the thyroid hormone to body functions is emphasized by the following summary:

1) It is necessary for normal growth and development of young animals. Young cattle deprived of their thyroid glands increase in bulk but not in height. They appear short-legged and have a broad, stocky build. Sexual development is also arrested.

2) It is necessary for the maintenance of the normal level of metabolism of animals of all ages.

3) It affects the development and the functioning of the nervous system.

4) The interrelationship between the thyroid and the gonads is indicated by the following observations:

(a) The thyroid is frequently observed to be enlarged at puberty and also during menstruation or pregnancy.

(b) In the dog and rabbit, castration frequently leads to a slow reduction in the size of the thyroid gland and a lowered metabolic rate.

(c) Rats and guinea pigs given continued injections of estrogenic substances develop an enlarged thyroid gland, followed in a few days by involution.

(d) Estrus is inhibited by feeding desiccated thyroid.

Asdell, writing for Dukes[7] on endocrine organs, states: "In several species it is now known that the level of thyroid activity, if it is varied within reasonable limits, does not affect the efficiency of the reproductive processes." He also writes: "Since the thyroid deficiency causes a lowering of basal metabolism, and hence a reduction in the efficiency of every cell of the body, many of the other hormones cannot exert their full effects unless the thyroid level of activity is adequate. The level of thyroxine secretion tends to be a limiting factor in many of the body processes."

That female reproductive performance can proceed normally only in euthyroid individuals has been shown in many experiments.[8, 11, 13, 14] The deleterious effect of hypothyroidism on embryonal survival and litter size has been shown by Krohn and White.[8] Hypothyroid rats produced only half as many young as normal controls. Thyroidectomized cattle do not come in heat.[13]

It is generally accepted that the iodine bound to serum proteins (PBI), rather than total iodine, more truly represents the level of circulating thyroid hormone and that practically all of the organic iodide in the blood consists of thyroxine.[2] Hence, the PBI may be used as an index of thyroid activity.

Bodansky[4] indicates that investigators have reported values of serum PBI ranging from 0.5 to 14.5 μg./100 ml. of serum for normal persons, with the vast majority between 3.0 and 8.0 μg. and an average value of about 5.0.

Kunkel *et al.*,[9] making a feeding experiment with beef cattle ranging in initial weight from 350 to 610 lb. (8 to 11 months of age), found the serum PBI to vary from 2.9 to 6.2 μg./100 ml. of serum. The average for a group of 10 Hereford bull calves was 4.28 μg., and the average for another group consisting of 11 Hereford and 12 Aberdeen Angus bull calves was 4.48 μg./100 ml. of serum. He found that calves with a wide variation in PBI had a wide variation in the rate of gain.

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Long *et al.*[10] made a comparison of the PBI values of 116 cattle representing several breeds. The average values obtained were: Jersey, 4.11 μg./100 ml.; Guernsey, 3.51; Brown Swiss, 3.37; Ayrshire, 3.19; Holstein-Friesian, 2.73; and beef breeds, 2.19. The extreme range was from 1.3 μg./100 ml. for a beef animal to 7.5 for a Jersey. The younger the animal, the higher was the PBI value.

MATERIALS AND METHODS

Blood samples were taken from 106 cattle in nine dairy herds. The PBI determinations were made as micrograms per 100 ml. of serum. Samples were taken from cows under a variety of circumstances. To overcome infertility, 2 cows were each fed 1 oz. of ethylenediamine dihydroiodide *

TABLE 1—Protein-Bound Iodine Values by Herds

Herd No.	No. blood samples	Av. PBI	Remarks
1	43	11.25	Johnson grass hay
2	14	8.14	Alfalfa hay
3	13	8.22	Alfalfa hay
4	3	5.6	Alfalfa hay
5	3	10.8	All sterile
6	5	6.5	Alfalfa hay
7	5	6.5	Alfalfa hay
8	8	4.0*	All aborted within 5 mo.; brucellosis; good nutrition
9	12	8.3	Good nutrition, alfalfa hay

* Statistically different from herd 1 ($p = .05$).

twice a day for one week before breeding. In these cases, the blood samples were taken two weeks before the start of treatment and immediately on termination of treatment.

The method of Chaney,[6] as modified by Moran,[12] was used to determine the PBI in the first series of samples. All samples were run in duplicate. The iodine-containing hormones were precipitated with the serum proteins using 10 per cent sodium tungstate and 0.67 *N* sulfuric acid. The protein precipitates were washed with water and digested with chromic and sulfuric acid. The iodine was oxidized to iodic acid, which remained in the digestant material while the other halides were removed by distillation during the digestion process. The iodic acid was then reduced and the iodine distilled off in a special distilling apparatus and collected in diluted alkali and arsenite. The iodine was then determined colorimetrically by its catalytic effect on the rate of reduction of ceric salts by arsenous acid.

The method described above was too intricate for routine clinical use. Careful boiling was required to expel chlorine and retain iodine, and only one sample could be run at a time. A method devised by Skanse and Hedenskog[15] has been modified and adapted for our use. This method consists of four steps: (1) precipitation and washing of serum proteins, (2) reduction to dry ash at high temperatures in the presence of alkali, (3) dissolution of the iodides from the ash by acids, and (4) colorimetric determination of the reaction velocity between arsenous acid and ceric sulfate, a reaction catalyzed by the iodides present in the dissolved ash.

(1) For precipitation and washing of the serum proteins, 1.0 ml. of serum was pipetted into a 40-ml. centrifuge tube and mixed with 2.5 ml. of zinc sulfate reagent (125 Gm. $ZnSO_4 \cdot 7H_2O + 17.5$ ml. conc. H_2SO_4 per liter of solution) and 20 ml. of double-distilled water. Three ml. of 0.75 N sodium hydroxide was added during the stirring. The tube was allowed to stand for at least three hours and was then centrifuged for 15 minutes at about 3,000 r.p.m. The precipitate was resuspended with 10 ml. of double-distilled water and recentrifuged. This procedure was repeated twice.

(2) In drying and incineration of the samples, the precipitate was transferred to a crucible with 2 ml. of 4 N sodium carbonate and 2 ml. of double-distilled water. The mixture was then dried overnight at 100 C. For incineration, the crucible was placed in an electric furnace for three hours at 625 C.

(3) To dissolve the iodides from the ash, 3.0 ml. of double-distilled water, 2.0 ml. of 2 N HCl, and 2.0 ml. of 7 N H_2SO_4, in this order, were added to the cooled incinerated sample.

(4) To obtain colorimetric determination of the iodide content, 3.0 ml. of the ash solution was mixed in a tube with 1.0 ml. of arsenous acid solution (3.71 Gm. As_2O_3 was dissolved in 50 ml. N NaOH, 200 ml. H_2O was added and the solution was neutralized with H_2SO_4. Then 54 ml. of 14 N H_2SO_4 was added and the solution made to 500 ml.). The mixture was allowed to stand at room temperature for about 30 minutes; 1.0 ml. of ceric ammonium sulfate (during stirring, 12 Gm. $Ce[SO_4]_2 \cdot [NH_4]_2\ SO_4$ was added to 500 ml. of 3.5 N H_2SO_4) was added and 30 minutes later the light transmission of the solution was read against distilled water in a Coleman Universal Spectrophotometer at 440 mμ. The amount of iodide present was determined with reference to a standard curve prepared with each series of determinations.

(5) The following method was used in preparation of standard curve:

1 ml. of standard containing:	Tube 1 0.04 μg. iodide	Tube 2 0.08 μg. iodide	Tube 3 0.12 μg. iodide
4 N Na_2CO_3	2.0 ml.	2.0 ml.	2.0 ml.
2 N HCl	2.0 ml.	2.0 ml.	2.0 ml.
7 N H_2SO_4	2.0 ml.	2.0 ml.	2.0 ml.

From each tube, 3.0 ml. was treated as described in (4) above.

* Hi-Amine compound (20 gr. ethylenediamine dihydroiodide per ounce in salt), available from Allied Laboratories, Inc., Indianapolis, Ind.

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RESULTS

The results are tabulated (tables 1–3). The over-all average PBI was 9.0 μg./100 ml. of serum. The average PBI value for all known pregnant cows (18) was 12.6 μg./100 ml. and for nonpregnant animals (88) the value was 8.6. This difference was statistically significant. The average for anestrous cows was 7.3 and for "repeat breeder" cows, 9.3 μg./100 ml. of serum.

TABLE 2—Protein-Bound Iodine Values Relative to Condition of Animals

Condition	No. cows	Av. PBI
Pregnant	18	12.6
Anestrous	9	7.3
Repeat breeder	10	9.3
Just calved	9	7.2
Pyometra	2	5.7
Before treatment*	2	6.9
After treatment	2 (same)	10.7
All animals	106 (samples)	9.0

* Ethylenediamine dihydroiodide, 20 gr./oz. in salt.

Herd 1, which had the highest average value, was on a ration of poor-quality Johnson grass hay and mineralized salt. About one third of these cows were pregnant, and their average PBI value was 14.8 as compared with an average of 9.8 for the nonpregnant cows in this group. The PBI values ranged from 3.5 to 18.3 μg./100 ml. of serum with 27 of 43 animals having a value of over 10.0. The increase in the PBI for this herd, which was not statistically significant, may have been due to a lower calcium content of the grass hay as compared with legume hay. High calcium rations have been reported to be goitrogenic.[16]

TABLE 3—P.B.I. Values Relative to Age of Animal

Age range	No. animals	Av. PBI
2 to 6 mo.	10	8.29
6 mo. to 1 yr.	7	8.8
1 to 2 yr.	33	11.9*
Over 2 yr.	56	7.64

* 12 of these animals were pregnant.

The lowest PBI average was in herd 8, where all cows examined had aborted and were infected with brucellosis. The difference between herd 1 and 8 was statistically significant; none of the other differences was significant. Since it has been established that hypothyroidism will cause abortion,[13] this fact should be kept in mind when considering the cause of abortions.

The number of animals given ethylenediamine dihydroiodide was too small for a statistical analysis. Further work should be conducted to study the effects of this organic iodide.

The differences in the PBI values as influenced by age were not significant. Two thirds of the animals between 1 and 2 years of age were pregnant. This factor was a cause for the high value of this group.

DISCUSSION

It has been shown that the PBI values for women increase 50 per cent during the last trimester of pregnancy.[2] The average PBI value for the pregnant cows during this period was 46 per cent greater than the average value for nonpregnant cows. The PBI values reported in this paper are considerably higher than some of the average values found in the literature. Since the methods used were thoroughly checked with known standards, there is no suitable explanation for the higher values obtained.

SUMMARY

Blood samples, taken from 106 dairy cows in nine herds under varying circumstances, were tested for protein-bound iodine (PBI). Values ranged between 2.1 and 18.3 μg./100 ml. of serum. Average values were listed relative to herd source, condition of the animals, and age.

The average PBI value for pregnant cows was found to be 46 per cent greater than that for nonpregnant cows. Those that had aborted as a result of brucellosis had significantly lower PBI values. There were no other indications that infertility was associated with abnormal thyroid function.

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Autoradiographic Studies of Normal and Leukemic Bovine Leukocytes

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IN RECENT YEARS, the advent of radioactive isotopes has given added impetus to the study of mammalian leukocytes. Numerous reports have appeared in the literature on the use of P^{32}-phosphate,[13,15] P^{32}-diisopropyl fluorophosphate,[1] C^{14}-formate,[21] C^{14}-adenine,[3,8] and C^{14}-thymidine[7,20] in the study of normal and leukemic leukocytes. The development of tritiated (H^3) thymidine,[18] as well as refinements of the microautoradiographic technique, has also provided a useful tool for the study of these cells *in vitro*[2] and *in vivo*.[5,9,10]

Thymidine has been shown to be incorporated exclusively into desoxyribonucleic acid (DNA) chemically[16] and isotopically[6,18] by rapidly dividing cells. Desoxyribonucleic acid occurs uniquely within the nuclei of cells associated with chromosomal material and remains there in an inert state;[19] *i.e.*, there is no intracellular renewal. New synthesis of DNA, however, has been observed in cells in mitosis at sites of high cell production in tissues.[20] Studies with plants[18] and bacterial cells[14] indicate a similar association of DNA synthesis with cell renewal. The synthesis of DNA by a cell is regarded as indirect evidence that the particular cell is in the process of renewal.

Therefore, a radioactively labeled compound such as C^{14}- or H^3-thymidine, which is incorporated solely into DNA, is stable, can be measured, and provides a means by which cell renewal systems can be studied. The measurement of radioactivity is conveniently performed by use of the microautoradiographic technique and, in addition, permits visual assessment of the individual cells which contain the label. Desoxyribonucleic acid sythesizing cells have been observed in the blood of normal human beings.[2] The present investigation was undertaken to study the blood of normal and leukemic cattle.

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MATERIALS AND METHODS

The normal animals used in this study were from a herd of purebred dairy cattle selected on the basis of history, physical examination, and hematologic findings. The diseased animals used were those from the same herd but either showed signs of leukemia or had a past history of hematologic findings highly suspicious of lymphocytic leukemia. Fifty milliliters of blood was withdrawn from the jugular vein into a heparinized syringe and transferred to a flask containing 100 μc. of tritiated thymidine. The tritium-labeled thymidine* had a specific activity of 350 mc. per millimole. The blood was incubated one hour at room temperature and then centrifuged ten minutes at 2,000 r.p.m. in an International type-2 centrifuge. The plasma layer was transferred to a 15-ml. centrifuge tube and centrifuged ten minutes at 3,000 r.p.m. The leukocyte-rich sediment was then transferred to a Wintrobe hematocrit tube and centrifuged ten minutes at 3,000 r.p.m. The buffy layer was next transferred to a 15-ml. centrifuge tube and washed once with 0.1 ml. of sodium chloride solution per millimeter of buffy layer. The cells were resuspended in sodium chloride solution, and smears made on 25- by 75-mm. microscope slides were fixed in absolute methyl alcohol. Coated autoradiographs were prepared by the dipping method of Messier and Leblond[12] using Kodak NTB-2 liquid emulsion.** The autoradiographs were exposed in a dry atmosphere in light-tight boxes for five days and then developed using Kodak D-19 developer, stop bath, and acid fixer. After development, the cells were stained with Giemsa stain through the emulsion, and cover slips applied prior to microscopic examination. A total of 5,000 cells was enumerated for each sample. In addition, a complete blood count, including a differential count of at least 200 cells, was done on a second sample of venous blood obtained from each animal at the time of sampling for the H^3-thymidine studies.

RESULTS AND DISCUSSION

Cellular detail and staining characteristics of the stained microautoradiograms were sufficient to permit the classification of the leukocytes into segmented leukocytes and small, medium, and large mononuclear

* Obtained from the Schwarz Laboratories, Mt. Vernon, N.Y.

** Obtained through the courtesy of the Eastman Kodak Company, Rochester, N. Y.

cells. The radioactive cells were also readily identifiable using the high-dry objective of the microscope; labeling was observed only in the mononuclear cells. An illustration of the appearance of a typical radioactive leukocyte is shown (fig. 1). Each labeled cell had at least 10 gr. directly over it, while background radioactivity was so low that corrections were unnecessary. These labeled cells were enumerated and the results summarized (table 1). The mean value and its standard deviation for the normal cattle was 1.03 ± 0.13.

Labeled cells were predominant among those classed as medium and large mononuclear cells, similar to the findings reported by Bond [2] for the blood of human beings. The findings show that in the peripheral blood of the cow, as well as that of man, there exists a small percentage of leukocytes capable of synthesizing new DNA. If it is a correct assumption that DNA synthesis implies the future division of a cell, then it may be possible, as suggested by others,[11] that medium and large lymphocytes are responsible for maintaining lymphocyte production. On the other hand, some authors [2] consider these labeled cells to be monocytic. The present studies make no differentiation between these cell types and therefore do not permit any conclusions to be reached in this respect. However, in view of the labeling observed in leukemic blood, it seems possible that there are more circulating cells capable of synthesizing DNA in leukemic cattle than in normal cattle.

It was of especial interest in this study that the 2 cows with a past history of hematologic findings highly suspicious of lymphocytic leukemia also showed a higher percentage of labeled cells. The differential leukocyte counts in these cows consistently showed a high percentage of prolymphocytes. It could well be that the labeled mononuclear cells observed in this study are derived from the primitive proliferating pool (PPP) of cells described earlier [4] in man and the mouse and variously termed stem cells, hemocytoblasts, and lymphoblasts. These PPP cells in man and the mouse have been observed in highest concentration in bone marrow, lymph nodes, and spleen and were shown to be in continuous migration throughout the body.[4] If the synthesis of DNA by a cell merely indicates a capacity for cell division, the requirement for a stimulus for cell propagation, as yet undescribed, is a possibility. It is known that the lymphocyte counts of cattle with lymphosarcoma show fluctuations during the course of the disease.[5,17] In periods of remission, even though a large number of cells capable of cell division

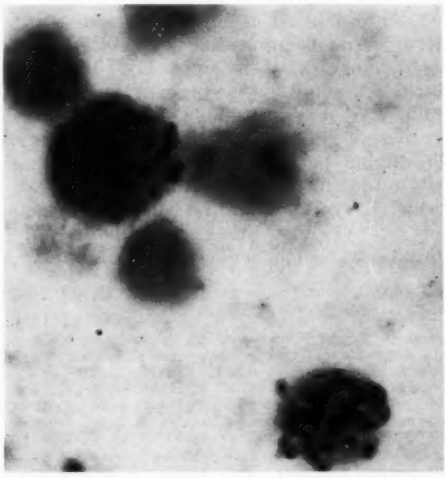

Fig. 1—Mononuclear cells in tritiated thymidine-labeled peripheral blood of a cow. x 1,200.

TABLE 1—Labeled Cells in the Leukocytes of Bovine Blood

Cow No.	Diagnosis	w.b.c. per cmm.	Differential count (%)			Total mononuclear cells labeled (%)
			Segmented leukocytes	Lymphocytes	Monocytes	
1	Normal	10,450	35.0	64.0	1.0	1.11
2	Normal	7,700	24.5	71.0	4.5	0.85
3	Normal	10,550	34.5	52.0	13.5	1.50
4	Normal	6,400	32.5	58.5	9.0	1.24
5	Normal	11,500	41.0	56.5	1.5	0.83
6	Normal	8,450	68.5	27.0	4.5	0.63
7	Suspect	7,850	17.0	75.0	8.0	1.49
8	Suspect	10,100	35.5	56.5	8.0	3.84
9	Leukemia	40,150	8.5	91.0	0.5	3.18

may be present, it is possible that high peripheral leukocyte counts are absent due to the lack of appropriate stimulus. The past history of these cows would indicate that, at the time of sampling, a large number of morphologically normal cells were biochemically dissimilar.

SUMMARY

Tritium-labeled thymidine was used *in vitro* for mieroautoradiographic studies of the leukocytes of normal and leukemic cattle. A small percentage of circulating leukocytes has been shown to be capable of synthesizing desoxyribonucleic acid (DNA). The higher percentage of DNA-synthesizing cells found in the circulating blood of cattle with lymphocytic leukemia indicated that many cells, though morphologically normal, were biochemically dissimilar.

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Further Observations on Immunity to Bovine Cutaneous Papillomatosis

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DURING the past several years a number of experiments have been done which relate to immunity against the common skin wart of cattle. The results of this work form the basis for this report.

The material is presented in five different sections: immunization achieved with single and multiple subcutaneous doses of bovine tissue vaccine; the immunizing effect of a growing papilloma and the influence of hyaluronidase on the immunity; immunization with live wart virus cultivated in chicken embryo; the concentration of bovine papilloma agent in epithelial and connective tissues of warts of various ages; and immunization with connective tissues containing wart virus and normal bovine skin. Specific details of the methods used, the results, and a summary are presented for each section. This is followed by a general discussion of the observations and an over-all summary.

MATERIALS AND METHODS

The Hereford calves * used were 6 to 9 months old, had been raised under range conditions, and were brought to feed lots prior to the beginning of the experiment. All calves were examined soon after they were received and in no instance were warts found. The calves were kept in lots of 10 each.

Bovine papilloma material containing the etiologic agent of the disease was stored in 50 per cent glycerin with water or physiologic saline solution and kept at about 4 C. The bovine papilloma agent has been found viable in such material after more than four years of storage. Three isolates or strains of bovine papilloma agent came from natural cases of the disease occurring on different farms. Two of these had been passaged serially on the skin of cattle, and the passaged material was used in some trials. The materials and strain they represent are indicated (table 1). A vaccine prepared from a material is designated by the material number.

IMMUNIZATION WITH SINGLE AND MULTIPLE SUBCUTANEOUS DOSES OF BOVINE TISSUE VACCINE

The vaccines were prepared by grinding bovine wart tissue with 0.8 per cent NaCl solution to make a 5 per cent suspension of tissue and adding formalin to a final concentration of 0.5 per cent. Two strains of bovine papilloma were used: strain 30 (material 212, representing strain 30 after two serial transfers on bovine skin) and strain 200 (harvested as warts from a natural case of the disease). These will be designated as vaccine 212 and vaccine 200 in the following discussion.

Five calves were each given a single 5-ml. dose of vaccine 212 and 5 others were each given a single 5-ml. dose of vaccine 200. Both vaccines were given subcutaneously. Five calves were given a series of six 5-ml. subcutaneous doses of vaccine 212 during an interval of two weeks, and 5 were similarly vaccinated with vaccine 200. The immunity of all 20 calves was challenged 62 days after the beginning of the vaccination schedule by duplicate intradermal injection and duplicate exposure of scarified skin to live wart virus in a ground suspension of bovine wart material. The three strains used for challenge were represented by material 200, material 212, and material 213. (Material 213 represented strain 207 after one passage on bovine skin.) Thus, each calf had 12 sites of exposure.

A group of 10 nonvaccinated, susceptible calves, obtained from the same source as the vaccinated calves, served as controls. Their immunity was challenged in the same manner.

RESULTS

The challenge sites were examined 34, 68, and 105 days after inoculation. In general, the reaction to intradermal inoculation was a nodular type of swelling in the

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This work was done in the Department of Animal Pathology and Hygiene, University of Nebraska, Lincoln. Data were compiled and the report prepared at the University of Wisconsin, Madison. L. V. Skidmore is associate professor emeritus, University of Nebraska. C. Olson and D. Segre are now in the Department of Veterinary Science, University of Wisconsin.

* Made available for experiments with warts through cooperation of the Department of Animal Husbandry.

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corium of the skin as a result of fibroblastic reaction with little epithelial proliferation. Reaction to inoculation in scarified skin was characterized by proliferation of the epithelium. None of the vaccinated calves showed a reaction at all sites of challenge. However, there were definite differences in size and number of the reactions among the groups of calves. All 120 exposure sites on the 10 control calves developed reactions which averaged 7.3 mm. in diameter.

The calves given multiple doses of vaccine 200 had the greatest resistance, in that 3-mm. reactions developed at only two of the 60 challenge sites. Reactions which averaged 3.75 mm. developed at eight of the 60 challenge sites in the 5 calves given multiple doses of vaccine 212. Some reactions occurred in each of the 5 calves. Three of the 5 calves given a single dose of vaccine 200 developed reactions at eight of the 60 challenge sites which averaged 3.7 mm. The 5 calves given a single dose of vaccine 212 developed reactions at 19 of the 60 sites of challenge exposure; the reactions averaged 5 mm. in diameter.

TABLE 1—Bovine Papilloma Materials Used in Experiments

Materials	Representing strains
30, 211, 212	30
200	200
207, 213	207

The 5 calves given multiple doses of vaccine 200 had no reaction to the subsequent challenge with material 200. This represents the only example of solid immunity against the homologous strain.

There was no evidence of a difference in resistance to intradermal challenge as compared to challenge on scarified skin; reactions occurred at 18 of the 120 intradermal exposure sites and 20 of the 120 scarified sites of exposure.

SUMMARY

A single dose of bovine wart tissue vaccine induced immunity. A greater degree of protection was provided by multiple doses as compared to a single dose in the case of material 212, but no difference could be shown with material 200. Bovine wart material 200 induced a greater degree of immunity than did material 212. These comparisons were found statistically significant at the 5 per cent level according to the probability tables of Mainland *et al.*[3] Since no attempt was made to assay the virus content of the two vaccine preparations, this may have been a factor. Both materials induced immunity against the other and also to a third and unrelated strain of bovine wart virus.

THE IMMUNIZING EFFECT OF A GROWING PAPILLOMA AND THE INFLUENCE OF HYALURONIDASE

Previous work [1,4] indicated that formalin-treated suspensions of bovine warts were capable of inducing a degree of immunity against a subsequent challenge with active papilloma virus. It was thought that a growing papilloma would provide antigenic stimulation of greater magnitude and longer duration than the formalinized vaccine and that the addition of hyaluronidase would aid the spread of antigenic material from the wart for better antibody production. An investigation of the ability of a growing papilloma to immunize against homologous and heterologous strains of virus, and of the influence of hyaluronidase on the immunity induced by actively growing warts, is the basis for this section.

MATERIALS AND METHODS

Six groups of 10 calves each were used for this experiment. Bovine papilloma virus contained in bovine wart tissue of materials 30, 200, and 213 was prepared by grinding tissue in enough 0.8 per cent NaCl to make a 10 per cent suspension. The three materials were each used to inoculate a group of 10 calves on a scarified skin area on the dorsal aspect of the neck immediately behind the horns. Three other groups of 10 calves each were similarly exposed, but skin was scarified on the right side of the neck and the material used for exposure of the scarified areas was prepared by mixing 2.5 mg. of hyaluronidase in 0.5 ml. of 0.8 per cent NaCl solution with 1.5 ml. of a 1:10 suspension of the respective bovine wart materials. In addition, 0.5 mg. of hyaluronidase in 0.1 ml. of 0.8 per cent NaCl solution was injected intradermally in the center of the scarified area at the time of exposure to bovine wart material.

Thus, each of the three strains of bovine wart material was inoculated into two groups of 10 calves each and calves in one of the groups were also given hyaluronidase.

The immunity that developed in the calves was challenged by exposure to the same three strains of bovine wart material. These were placed intradermally and on scarified skin 71 days after the original exposure.

RESULTS

Although there was some variation in the response of the calves to the original inoculation, only two of the 60 calves failed to develop warts. The variability of reaction in individual calves is illustrated by the graphic records of the 10 calves given strain 30 material with hyaluronidase (fig. 1). The response varied from that observed in 1 relatively resistant animal which developed a 5-mm. papilloma at 71 days that completely regressed 35 days later, to the development in 1 animal of a wart which reached the size of 8 cm. at 105 days, was present 72 days later, and was not completely regressed until 260 days after its inoculation. Such variations as illustrated in figure 1 are quite typical of the reactions to be expected with any group of susceptible calves.

Hyaluronidase had no influence on the growth or regression of the warts produced from the original exposure (fig. 2). Of the 60 calves, 37 had no reaction from the challenge inoculations. This indicated a solid immunity in these animals, since all 10 calves of a control group (no previous exposure to bovine papilloma material) developed reactions from all three strains of the challenge materials at both scarified and intradermal sites of exposure.

Four calves developed reactions at all of the sites of challenge exposure, but these were smaller and of shorter duration than occurred in the control calves. Results similar to that portrayed for 1 calf (fig. 3) were obtained in 3 calves. The fourth calf which developed a reaction from the challenge exposures was unusual in that growth from the immunizing inoculation was not evident until 139 days after exposure and was simultaneous with the development of the challenge exposure (fig. 4). This was the only animal of 58 calves to manifest such a delayed response to the immunizing dose of bovine papilloma material (strain 30 material without hyaluronidase). The response of this calf to inoculation by scarification with challenge materials was greater than the average of 10 control calves.

The remaining 19 calves which developed some reaction from the challenge exposures, thus indicating a relative lack of immunity, showed a considerable variation in the growth pattern from the challenge inoculations. Some had only a small reaction at

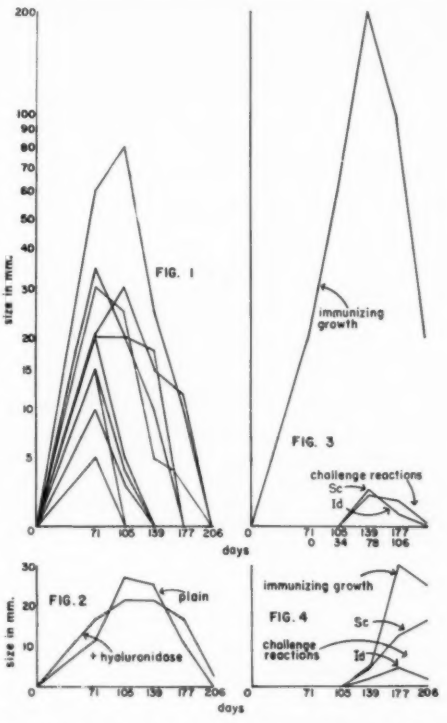

Fig. 1—Growth curves of papillomas developed in 10 calves as a result of exposure of scarified skin to bovine papilloma agent mixed with hyaluronidase. The lines illustrate the range of individual reactions usually obtained with a group of susceptible calves and do not demonstrate any peculiarity of this group or an influence of hyaluronidase.

Fig. 2—Average growth of curve of warts produced in 30 calves (three groups of 10 each) with three strains of bovine wart material and the growth curve of warts in 30 calves (three groups of 10 each) induced with the same three strains of bovine papilloma material to which hyaluronidase was added. Essentially, hyaluronidase had no influence on growth of the warts.

Fig. 3—An example of the average growth curves of reactions at sites of challenge exposure in a calf with a bovine papilloma (200 mm.) induced 71 days prior to the challenge of immunity. Three of 60 similarly treated calves had this type of response. Average reaction of the challenge materials in 10 other calves is illustrated for comparison (Id = intradermal challenge; Sc = scarification).

Fig. 4—An unusual delayed reaction to an immunizing dose of active bovine papilloma agent and simultaneous exaggerated reaction to challenge materials inoculated on scarified skin.

one or two of the 12 sites of challenge exposure (the 3 challenge strains were injected intradermally and on scarified skin, and each type of exposure was done in duplicate). Many of the reactions were so small they would have been unnoticed with only a casual observation.

Hyaluronidase had no influence on the subsequent immunity induced by papilloma as a result of inoculation with materials 200 and 213. Seven of 10 calves given strain 30 material plus hyaluronidase were subsequently immune to the challenge exposure, whereas 3 of the 10 calves given strain 30 material alone to induce immunizing papilloma were immune to the subsequent challenge. None of the partially susceptible calves given strain 30 material without hyaluronidase had reactions to challenge materials at all sites of exposure in the manner described previously. These results with respect to the influence of hyaluronidase on the subsequent immunity were not statistically significant.

SUMMARY

Actively growing bovine papilloma induced solid immunity in the majority of 60 test calves by 71 days after the immunizing inoculation. Hyaluronidase was without significant influence on the development of immunity. Four of the 60 calves (approximately 7%) were distinctly susceptible to the challenge inoculations even though all had actively growing warts. The degree of susceptibility, however, was somewhat less than in control animals. One calf exhibited an unusual delay in response to the dose of bovine papilloma agent used for immunizing. Hyaluronidase did not influence the growth curve (size or duration) of experimentally induced papillomas.

IMMUNIZATION WITH LIVE WART VIRUS CULTIVATED IN CHICKEN EMBRYO

Previous results [1] indicated that commercial wart vaccine prepared in chicken embryos was ineffective in producing immunity to a subsequent challenge. This was also true when the strain of challenge agent was the same as that used for immunization.[4]

This raised a question of the degree to which the agent of bovine warts could grow in chicken embryos. Therefore, test calves were given material from chicken embryos that had been inoculated with bovine wart agents. Some of the calves developed warts and the immunity of all was challenged with active, wart-producing materials.

MATERIALS AND METHODS

Strains 30, 200, and 207 of bovine wart virus contained in bovine wart materials 30, 200, and 213 were carried on individual serial transfers for nine passages on the chorioallantoic membranes of embryonating chicken eggs maintained at 38 C. At the time of inoculation the eggs had embryonated 10 days. The membranes were harvested five days later. At the ninth serial passage, sufficient membranes were harvested to provide an inoculum for the test calves. The membranes were ground and diluted with 10 parts of sterile physiologic saline solution.

Chicken embryo material containing strain 30 was given to 10 calves in one group. The material was applied to a single scarified area approximately 0.5 inch in diameter on the top of the neck just back of the horns. Two other groups of 10 calves each were similarly inoculated with chicken embryo material containing strains 200 and 207, respectively.

The degree of immunity resulting from growths or exposure to wart virus grown in chicken embryos was measured by a challenge exposure 71 days later. For challenge, active bovine papilloma virus contained in bovine wart materials 30, 200, and 213 was used. These materials were inoculated in duplicate on the skin of the neck both on scarified epithelium and intradermally.

Observations were made on the reactions at varying intervals of time.

Ten calves not previously exposed to any type of bovine papilloma material were given the challenge inoculations at the same time as the 30 calves in the immunization experiment.

RESULTS

One of the 10 calves given strain 30 in chicken embryo developed a wart 5 mm. in diameter which appeared 139 days later and remained for only 30 days. There was no reaction in the other 9 calves. Upon subsequent challenge of immunity, 8 of the calves developed reactions. In 5 calves the reaction was of the same degree as that in the controls, and in 3 calves the reaction was more marked than in any of the controls (1 of these was the calf which developed a wart from the chicken embryo material). One calf had a slight reaction from the challenge with strain 200 material and the tenth calf was completely negative to the challenge materials.

Two of the 10 calves given strain 200 material in chicken embryo developed warts at the scarified sites at 139 and 177 days; warts remained for only a short time.

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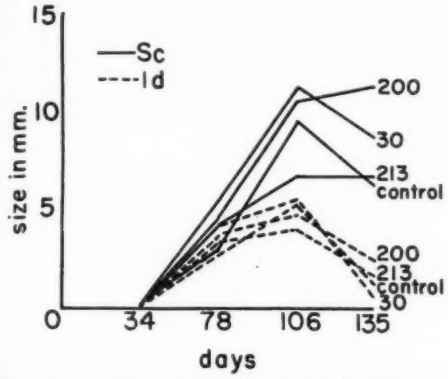

Fig. 5—Duration and size of reactions to challenge of calves 71 days after exposure to wart virus grown in chicken embryos. Each line represents the average of reaction obtained in 10 calves with three strains of virus in bovine tissue used for challenge. The numbers 200, 30, and 213 identify the immunizing material. Solid lines represent growth curve of warts at sites of scarification (Sc) and dotted lines at sites of intradermal (Id) injection.

Upon challenge of their immunity, 3 of the 10 calves had a more marked reaction than any of the controls, 3 had slightly more reaction than the controls, and 3 had the same reaction as the average of those of the controls. The tenth calf had less reaction.

Two of the 10 calves given strain 207 in chicken embryo material developed 5-mm. warts which appeared at 139 days and lasted for a little longer than 30 days. Upon challenge, 2 of the calves had reactions more marked than any of those of the controls, 2 slightly more than the average in the controls, and 3 about the same as in the controls. Three calves had reactions less marked than those of the controls.

The reactions in the 10 controls were quite uniform. The average size and duration of the reactions are indicated (fig. 5).

The reaction from bovine wart virus contained in the chicken embryo material was relatively late in its appearance and was first noticed 139 days after exposure in 5 of the calves exposed. Appearance of the reaction was simultaneous with the first evidence of growth from the challenge inoculations (fig. 6).

Summary

There was no evidence of immunity developing as a result of the previous exposure to live wart virus contained in chicken embryo material. The reaction to intradermal injection of challenge materials was the same as in the controls. There was some variation of the reaction at the site of challenge on scarified skin; 8 of the 30 calves had a more marked reaction than occurred in the controls.

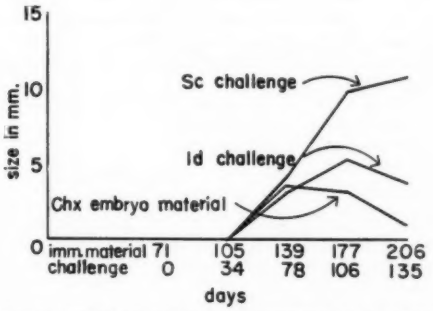

Fig. 6—Duration and size of reactions in 5 calves which developed warts from bovine wart virus grown in chicken embryo and also from the subsequent challenge inoculations. Notice the long delay of reaction from wart virus in chicken embryo material inoculated 71 days prior to challenge inoculations.

Response to chicken embryo material developed in each of the three groups of calves indicating that wart virus did in fact exist in the ninth passage of chicken embryo culture. Since it provoked reactions in only 5 of 30 animals and was late in development, it would seem that the concentration of virus in the inoculum was low, or that the character of the virus may have been altered by residence in chicken embryo tissue.

Concentration of Bovine Papilloma Agent in Epithelial and Connective Tissues of Warts of Various Ages

It was desirable to know whether the bovine papilloma agent was present in different concentrations in the different parts of a wart, or in warts obtained at different times after inoculation.

Materials and Methods

Two strains of bovine papilloma agent were used in this experiment. Material 211 was a wart excised 60 days after inoculation of strain 30 into the scarified skin of a calf. Material 212 was a wart from the same calf but was obtained 120 days after exposure. Material 213 was the growth obtained 120 days after exposure of a

calf to papilloma virus (strain 207) from a natural case of the disease, and represents a different strain of agent.

These warts were dissected into epithelial and connective tissues, and each part was ground and diluted with 0.8 per cent NaCl solution to obtain dilutions from 10^{-2} to 10^{-6} on the basis of the tissue weight. Each dilution was then inoculated in duplicate on the scarified skin of the necks of 5 calves. The same 5 calves were used for materials 211 and 212, which were inoculated each on one side of the neck. The dilutions of material 213 were inoculated on one side of the necks of 5 more calves.

The inoculation sites were observed one, two, and three months after inoculation and the sizes of the reactions were recorded.

RESULTS

The concentration of papilloma virus in the different materials inoculated was calculated in terms of 50 per cent infectious dose ($i.d._{50}$), using the formula of Reed and Muench [7] (table 2). One $i.d._{50}$ is defined as the amount of papilloma virus which causes 'takes' in 50 per cent of the sites inoculated. The infectivity titers so expressed do not therefore convey any information as to the size of the warts resulting from inoculation of different amounts of virus. In general, however, an inverse relationship was noticed between virus dilution and size of growth, the lower dilutions resulting in the larger growths.

TABLE 2—Infectivity Titers (in $i.d._{50}$ per Inoculation Dose) of Epithelial and Connective Tissues from Warts of Various Ages

Material	Origin	Age of warts in days	Titer (log $i.d._{50}$)	
			Epithelium	Connective tissues
211	Strain 30	60	4.08	3.63
212	Strain 30	120	5.44	4.37
213	Field case	120	5.32	5.79

Significantly higher virus concentrations were found in the 120-day wart than in the 60-day wart. The concentration of strain 30 (material 211) virus was higher in the epithelium than in the corium of both 60- and 120-day warts. On the contrary, the virus derived from material 213 was more abundant in the connective tissue than in the epithelium. Whether or not this finding is a reflection of a strain difference cannot be decided until more experiments of this same kind are conducted with a number of virus strains.

SUMMARY

Infectivity titrations were performed with epithelial and connective tissues from 60- and 120-day-old warts obtained from 1 calf and produced by one strain of bovine papilloma virus, and from a 120-day-old wart derived from another strain of virus. Higher concentration of virus was found in the 120-day-old warts than in the 60-day-old one. With one strain the virus was more abundant in the epithelium, with the other it was more abundant in the connective tissue.

ATTEMPTED IMMUNIZATION WITH CONNECTIVE TISSUE CONTAINING WART VIRUS AND NORMAL BOVINE SKIN

The purpose of these experiments was to test the immunizing properties of preparations suspected of containing a masked wart virus. In a preliminary experiment the affected epithelium and connective tissue from the corium of a naturally occurring wart were separated by dissection and these portions (material 207) were stored separately in 50 per cent glycerin. Two months later, serial dilutions of each material were prepared in ground suspension and inoculated in the skin of a test calf. The epithelial portion was still infectious in a dilution of 10^{-6}, while the connective tissue was not infectious in the lowest dilution tried (10^{-2}). It was thought that the virus in the connective tissue might be in a masked, nonvirulent form and that it might be detected by its immunizing capacity. Previous experiments had also indicated that bovine papilloma virus, when passaged in the horse, could not regularly be transferred back to cattle. It was thought that, in that instance also, the virus might be in a masked state and could be demonstrated by its capacity to immunize.

Twelve calves were given corium of bovine wart material 213 (1 bovine passage of material 207). The wart had been stored for about 10 days in 50 per cent glycerin when the corium was separated from the epithelial portion by dissection. The portions of corium were washed by holding them under running tap water to remove glycerin solution which might contain traces of virus. A 5 per cent suspension of the corium was prepared and antibiotics (1,000 units of penicillin and 1 mg. of streptomycin per ml.) were added to control bacterial contamination. The prepara-

tion of wart corium was inoculated onto scarified skin of the 12 test calves and, in addition, two 5-ml. doses were injected subcutaneously, one week apart, into each calf to provide a greater amount of immunogenic material.

Each of the 12 calves developed a wart at the site of inoculation with corium suspension; the average maximum diameter was 6.5 mm. Sixty days after this inoculation the immunity of the 12 calves was challenged by both intradermal and scarified skin exposure to bovine wart virus contained in material 30 and 213. All of the calves were susceptible to the challenge exposure (fig. 7).

ATTEMPTED IMMUNIZATION WITH BOVINE WART VIRUS CONTAINED IN THE CORIUM OF SKIN FROM THE HORSE

The corium of the horse will respond to bovine wart virus by a rapid proliferation of fibroblasts whose growth will resemble a fibrosarcoma, and yet the reaction is self-limiting. Since no information was available on the probable concentration of wart virus in such reactions, a 5 per cent composite suspension of 3 such "sarcomas" was prepared. These had been biopsied 18, 29, and 50 days after intradermal inoculation of a horse with strain 30 material.

The suspension of equine "sarcoma" was inoculated onto scarified skin of 12 test calves and two 5-ml. doses were injected subcutaneously into each calf. Four of the 12 calves developed warts with an average diameter of 6 mm. (fig. 7). The immunity of all 12 calves was challenged by exposure to active bovine wart virus contained in material 212 (strain 30) and material 213 (strain 213). Of the 12 calves, 8 were susceptible and demonstrated no immunity. Two of the 4 calves which developed warts from the immunizing exposure were susceptible to the challenge exposures.

ATTEMPTED IMMUNIZATION WITH NORMAL BOVINE SKIN

As a control experiment, 13 calves were each given two 5-ml. subcutaneous injections of a 5 per cent suspension of normal skin which was taken from a calf and contained 0.5 per cent formalin. The two injections were given one week apart. Sixty-five days after the first injection, the immunity of the calves was challenged by exposure to the same materials and in the same manner as in the previous experiments. Twelve of the 13 calves were susceptible; the average size of the reactions is indicated (fig. 7).

SUMMARY

Active wart virus capable of producing reactions was present in the corium of a bovine wart and also present in the connective tissue proliferation caused by bovine wart virus in the skin of a horse. In calves, neither of these two materials produced any significant immunity to subsequent inoculation with active bovine wart material.

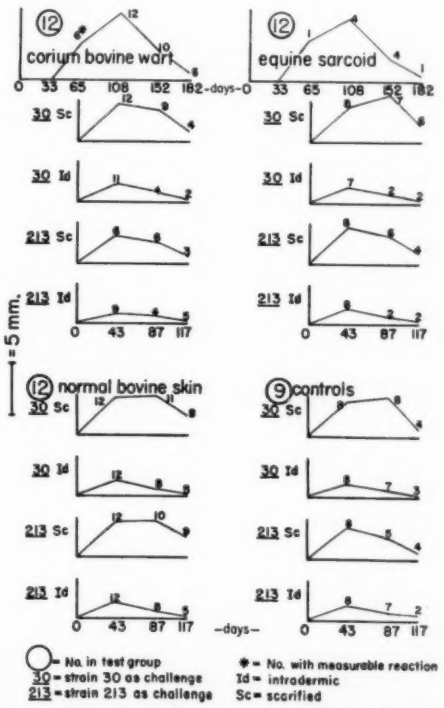

Fig. 7—Graphic presentation of growths obtained in 12 calves which received corium of bovine wart (upper left) and reactions to subsequent challenge exposure (four graphs immediately below); in 12 calves which received experimentally produced equine sarcoid material (upper right) and reactions to subsequent challenge exposure (four graphs immediately below); the reactions of 12 calves which had previously received normal bovine skin (four graphs in lower left area) and reactions of 9 control calves (four graphs in lower right area). The similarity of reactions to the various challenge exposures in the four groups of calves shows lack of immunity from the warts produced by the corium of bovine wart or the tissue of an equine sarcoid produced by bovine wart material. Normal bovine skin was not expected to produce immunity.

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NONVACCINATED CONTROL CALVES

Nine calves, not previously vaccinated, were exposed at the same time and to the same materials as calves described in the three preceding sections. Eight of 9 were susceptible. The average size of their reactions is indicated (fig. 7).

DISCUSSION

The results support previous observations that material containing bovine wart virus will immunize cattle against a subsequent exposure.[1,2] One of the experiments indicated that a single dose of a formalinized suspension of bovine wart tissue would produce a significant degree of immunity. Two strains of bovine wart material were used in these trials, and one (material 200) produced a slightly greater degree of immunity. Both of these strains of bovine wart induced immunity against the other and also against a third unrelated strain to apparently the same degree. The immunity status of the animals was challenged with the two strains used to induce the immunity and also with a third unrelated strain. In previously reported experiments,[1] differences in response to challenge with three strains of wart material suggested the existence of antigen types; these differences were not observed in the current trials.

It was found that a single actively growing wart would induce resistance to experimental reinfection in most of the animals. The immunity status was challenged 71 days after the initial infection that established the growing wart. At this time the growing wart was at about its maximum size (fig. 1). The addition of hyaluronidase to the initial infective material was intended to aid the spread of antigenic materials from the wart, but it failed to induce better immunity. Hyaluronidase did not influence the growth of the wart which was induced. This experiment also illustrates the wide variation of individual calves in their reaction to the wart agent as well as in their immunologic response. Of the 60 calves in the experiment, 58 developed a wart. About 7 per cent of the 58 calves which had actively growing experimental warts were distinctly susceptible to challenge exposure. About 32 per cent developed slight reactions from the challenge exposures, indicating only a partial degree of immunity. About 61 per cent of the calves demonstrated solid immunity by having no reaction to the challenge inoculation.

Rosenberger[8] obtained more clean-cut results with a smaller group of animals. He used 8 cattle, 12 to 18 months old, 2 of which had recovered from natural infection, 2 from experimental infection, 2 in which the warts had been removed surgically, and 2 controls. Their immunity status was challenged by infection of scarified skin with fresh wart material. Only 2 controls developed the disease. The marked variation in the reaction of individual calves to the same wart inoculum is illustrated (fig. 1). These variations in both reaction to the agent of bovine papillomatosis and development of immunity indicate the need for using several calves in experimentation. The partial degree of immunity of some calves was also observed in previous experiments.[1,4]

In one series of experiments, the test calves were first injected with bovine wart agent which had been maintained in chicken embryo cultures and presumably multiplied there. Nine serial pasages were made in chicken embryos to eliminate the possibility of dilution from the original wart inoculum. Since only a few of the calves developed a wart at the site of injection, and these warts were small and very late in developing, it can be assumed that active wart virus was either quite low in concentration or altered in infectivity. The chicken embryo material did not induce resistance to subsequent challenge inoculation with wart virus in suspensions of bovine warts.

Infectivity titrations of the epithelial and connective tissue portions of warts indicated a greater concentration of active virus in each of two 120-day-old papillomas than in one 60 days old. There was some variation of concentration of active virus in epithelium as compared to connective tissue, but this variation was not consistent in two trials with two different strains of wart agent.

A most peculiar result was obtained when using connective tissue of the corium of a bovine wart and a connective tissue from a "sarcoma" caused by bovine wart virus in the skin of a horse. An agent capable of producing warts was present in the connec-

tive tissues of both types of lesions. The warts produced by the agent in the corium (connective tissue) of a bovine wart and supplemented by two 5-ml. subcutaneous doses of the same material did not produce immunity to challenge 60 days later, indicating an alteration of the immunogenic character of the wart agent in the connective tissue of a bovine wart. A similar suggestion of a change in the immunogenic character of bovine wart agent was found in a "sarcoma" lesion of a horse. A suspension of the equine lesion was inoculated on scarified skin of calves, each of which also received two 5-ml. subcutaneous doses of the same material. Warts developed in 4 of the calves. Two of these 4 calves with warts and 6 others of the group were susceptible to subsequent challenge with active bovine wart material. The significance of these observations is not clear at this time.

A formalinized suspension of normal bovine skin given in two subcutaneous injections produced no evidence of immunity in calves challenged with active bovine wart material. The challenge exposures were made 65 days after the injections of normal bovine skin.

Evidently none of the calves had been exposed to wart agent prior to their use in the experiments reported, since none of them had warts when they were obtained, and with rare exceptions they developed warts only where infective material had been inoculated. These rare exceptions were cases in which warts developed at sites not inoculated and appeared late in the experiment. They probably arose by spread from the experimentally induced warts and did not influence the results presented. Unlike calves raised on a range, those calves raised under ordinary farming conditions have more intimate contact with cattle of various ages, and warts have been observed in such calves when about 1 month old. It is reasonable to assume that the test calves represented a fairly uniform population in which there were animals with the inherent variations of susceptibility which had not been affected by previous exposure to the agent of bovine papillomatosis.

The immunity phenomena in bovine cutaneous papillomatosis appear to be complex. The recent reports by Pearson *et al.*[6] and Steele-Bodger and Wright[9] indicate successful use of tissue vaccines in the treatment of bovine papillomatosis under the conditions of farm practice. Under experimental conditions, Olson and Skidmore[5] found that administration of a vaccine caused no appreciable regression of experimentally induced warts.

SUMMARY

Test calves were subjected to a number of vaccination treatments intended to immunize them against subsequent challenge with active bovine papillomatosis materials. A single injection with a formalinized suspension of bovine wart material produced a significant degree of immunity. A single actively growing wart induced immunity to challenge with the bovine papilloma agent in nearly two thirds of the calves. Nearly one third had a partial degree of immunity, and a few remained fully susceptible.

The agent of bovine papilloma propagated in chicken embryos had a low degree of infectivity and no immunizing ability for calves.

It appeared from a few trials that there was a higher concentration of agent in warts 120 days old than in a wart 60 days old. The papilloma agent was found in both the epithelia and the connective tissues of bovine warts.

The connective tissue from the corium of the bovine wart and the connective tissue of a "sarcoma" caused by bovine wart virus in the skin of a horse contained virus capable of producing warts in calves. Calves given the infected connective tissue material remained susceptible to the bovine papilloma agent.

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A Virus of Ovine Abortion—Isolation from Sheep in the United States and Characterization of the Agent

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A VIRUS of the psittacosis-lymphogranuloma venereum (PL) group was described as the cause of enzootic abortion of ewes in 1950 by Stamp *et al.*[19] in Scotland. In 1951, Spanedda and Medda[17] cultivated, from aborting sheep in Sardinia, a virus which may have belonged to this group. In 1954, Mitscherlich[12] isolated a PL virus from similarly affected ewes in Germany. This was repeated in 1956 by Giroud *et al.*[4] in France, and in 1958 by Hajdu *et al.*[6] in Hungary.

In the spring of 1958, virus abortion of ewes had not been recognized as prevalent in the United States, and the PL group viruses known to occur here were not known to cause ovine abortion. At this time, Young *et al.*,[23] at the Montana Veterinary Research Laboratory, reported the presence of ovine virus abortion in northwestern United States which clinically and epidemiologically resembled enzootic abortion of ewes. The purpose of this study is to describe the isolation and characterization of a virus from these infected sheep and to compare it with the members of the PL group, especially the virus of enzootic abortion of ewes (EAE).

MATERIALS AND METHODS

Staining of Smears.—After being fixed by heat, smears were covered with a strip of filter paper and stained by Stamp's modified Ziehl-Neelsen method.[19] Ziehl-Neelsen carbol-fuchsin, diluted 1:5, was applied for ten minutes. Stain and paper were tipped off, the slides were dipped in 0.5 per cent acetic acid in a Coplin jar for two to three seconds, washed gently with water, and stained with 1 per cent aqueous methylene blue for 20 seconds.

Preparation of Tissues for Inoculation.—Tissues were ground in a sterile mortar, a Tenbroeck grinder, or in an Omni-Mixer * and diluted about 1:5 in hormone broth of pH 7.4. Tissues contaminated with bacteria were diluted in a tyrothricin, sulfadiazine, and streptomycin (TSS) solution.[11] Suspensions were allowed to remain at room temperature for 30 minutes during which they were centrifuged ten minutes at 1,500 r.p.m. to remove tissue particles.

Inoculation and Examination of Eggs.—Tissue suspensions (0.5-ml. volumes) were inoculated into the yolk sacs of groups of 5- to 7-day embryonating chicken eggs. They were candled daily for the next 13 days. Embryos found dead during the first 48 hours were discarded. Yolk sacs of those found dead during the remainder of the observation period and those surviving on the thirteenth day were smeared and examined for elementary bodies.

Virus Titration in Eggs.—Tissues to be titrated were weighed, and tenfold dilutions were prepared and inoculated into the yolk sacs of groups of 4 to 6 eggs. Deaths were considered due to infection only when elementary bodies were demonstrated. Insofar as possible, dosages used in this work were expressed as 50 per cent embryo lethal doses ($e.l.d._{50}$) per 0.5 ml. of inoculum. This value was calculated by the method of Reed and Muench.[16]

Virus Strains.—The virus being investigated in this paper is a strain isolated (as described in the next section) from the lung tissue of an aborted sheep fetus. The EAE virus** used in these studies was a strain isolated by Dr. J. T. Stamp of the Veterinary Investigation Center Laboratory, Edinburgh, Scotland. It is referred to as the Stamp strain. The virus of sporadic bovine encephalomyelitis used was islolated in this laboratory from a calf showing typical signs of the disease.

EXPERIMENTAL PROCEDURE AND RESULTS

ISOLATION OF VIRUS

Isolation in Embryonating Eggs.—Aborted fetuses and placental membranes from flocks of sheep in which no bacterial agents of abortion could be found were used for isolation of virus. Elementary bodies were usually found in direct smears from cotyledons or membranes but not from fetal organs. Under X900 magnification they appeared as barely visible red or blue spheres on a blue background. In some

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* Ivan Sorvall, Inc., Norwalk, Conn.

** Culture supplied by Mr. R. K. Geroff, National Microbiological Institutes, Rocky Mountain Laboratory, Hamilton, Mont.

preparations all of them appeared to be red, while in others both red and blue particles were seen. Blue spheres alone were not recognized as virus. Bacteria, which were frequently numerous, stained red or blue but were distinguishable by their difference in size, shape, and arrangement. Elementary bodies were often numerous in placental tissue, where they occurred both extra-cellularly and within epithelial cells, and mononuclear and polymorphonuclear leukoytes. In some cells they were concentrated in circumscribed areas, while in others they were irregularly scattered through the cytoplasm. Isolated globules consisting of masses of elementary bodies were frequently seen.

Suspensions prepared from infected tissues were inoculated into the yolk sacs of embryonating eggs. On being examined at the time of death of the embryo or termination of the incubation period, the yolk sacs usually showed elementary bodies dispersed throughout the smear and often very numerous. The use of fetal lung, liver, spleen, or kidney tissue for isolation of the virus avoided some of the contamination encountered with placental tissue but involved inoculating a larger number of eggs, since the concentration of virus in these organs was low. Deaths in first-passage eggs occurred between four and 13 days after injection. A few infected embryos survived. If yolk sac smears from embryos which had died showed no elementary bodies, it was possible in some instances to demonstrate the virus on second passage.

By this method, a virus was cultivated from three aborted placentas and the lung of one aborted fetus obtained from three separate flocks of sheep. In addition, elementary bodies were found in smears from ten fetal membranes obtained from four flocks.

TABLE 1—Survival Times of Eggs Inoculated into the Yolk Sac with Mixtures of M Virus and Antibiotic and Chemotherapeutic Agents

Antibiotic	Dose per egg	No. of eggs	Av. day of death*
p-Aminobenzoic acid	20 mg.	7	5.4
Penicillin	2,000 units	5	11.6
Streptomycin	10 mg.	6	5.5
Sulfadiazine	2 mg.	4	6.0
Sulfathiazole	10 mg.	5	11.6
Untreated controls	0 mg.	5	5.8

* Considering the data in 2 by 2 contingency tables where the survival dichotomy is seven days or less, the exact test shows significance at the 1 per cent level between the survival times of the controls and both penicillin and sulfathiazole treated groups. There was no significant difference between the other antibiotics and the controls.

ATTEMPTED CULTIVATION IN NONLIVING MEDIUMS

To test the ability of the elementary bodies to multiply in the absence of living cells, heavily infected yolk sac was inoculated into various bacterial mediums commonly used for the cultivation of fastidious organisms and incubated at 37 C. for seven days. The cultures were titrated in eggs before and after incubation. They showed no increase in virus content.

This result to some extent confirmed the impression that the agent isolated was either one of the Rickettsiaceae or a virus of the PL group. It will be referred to as M virus in discussing experiments on its characteristics and identity.

STABILITY OF THE VIRUS

Resistance.—In tissues, M virus was found to remain viable for at least two days at room temperature and for several days in a refrigerator. Viability of suspensions was reduced by freezing and thawing.

Inhibition by Antibiotic and Chemotherapeutic Drugs.—The effect of certain antibiotic and chemotherapeutic drugs on the production of infection in the yolk sacs of embryonating eggs was tested. A 0.2-ml. amount of infected yolk sac suspension containing about $10^{6.5}$ e.l.d.$_{50}$ of virus was mixed immediately before inoculation with 0.5 ml. of distilled water containing the indicated amounts of antibiotic. At the dose level used, no inhibition of viral multiplication was shown by *p*-aminobenzoic acid, streptomycin, or sulfadiazine (table 1). Penicillin and sulfathiazole both showed statistically significant inhibition.

HOST SPECTRUM

Pathogenicity for Sheep.—The clinical disease in ewes and the pathologic changes produced have been described.[23] In infected flocks, no clinical signs were observed in any sheep other than pregnant ewes. In these animals, the virus had a tropism for placental tissue. The e.l.d.$_{50}$ titers of portions of placental tissue varied from $10^{-2.3}$ to $10^{-6.0}$. Serums taken near the time of

abortion usually showed complement-fixation titers of 1:32 to 1:128 against heated M virus antigen.

Growth in Embryonating Eggs.—Inoculation into the Yolk Sac of Embryonating Eggs.—The M virus exhibited characteristic PL tropism for yolk sac tissue of embryonating eggs. Concentrations of $10^{9.5}$ e.l.d.$_{50}$ per 0.5 Gm. were frequently found in this tissue. Serial passage was readily accomplished. Neither the titer of virus attained nor the time required for death of the embryo appeared to change after six passages.

Embryos which died after inoculation with M virus showed a retarded development when compared with normal controls. The body of such an embryo was smaller and less feathered than normal. The yolk was similar to those of younger normal eggs, in that it retained to a considerable extent its thin consistency and bright yellow color. As hatching time approached, the thick, dark yolk of the normal egg could not readily be removed from the degenerating yolk sac, while infected yolk sac usually showed little or no degeneration and was readily freed from yolk. The skin of the embryo dead from infection was deep red due to hyperemia and slight hemorrhage, and the yolk sac vessels were congested. Embryos surviving 13 days after inoculation were sometimes heavily infected though grossly normal.

With a number of viruses of the PL group, Golub [5] demonstrated the existence of a linear relationship between concentration of virus inoculated into the yolk sac and survival time of the embryos. In order to determine whether such a relationship existed for the M virus, data from several titrations were plotted with the negative log of the dilution against the average day of death of the embryos. A straight line relationship between these values was indicated. While the data used were insufficient to determine the exact value, the slope of the lines for all titrations of M virus was less steep than reported for other members of the group.[5, 22] This indicated a less rapid growth of M virus in the yolk sacs of embryonating eggs.

Inoculation into the Allantoic Cavity of Embryonating Eggs.—After inoculation of 10^6 to 10^7 e.l.d.$_{50}$ of yolk sac virus into the allantoic cavity of 9-day embryonating eggs, some embryos died between the fourth and the tenth day. Pooled fluid from inoculated eggs had an e.l.d.$_{50}$ titer of $10^{-5.5}$ after freezing.

PATHOGENICITY FOR MICE

Intranasal Inoculation.—The M virus was found to possess the pneumotropism characteristic of members of the PL group, attaining concentrations of $10^{8.5}$ e.l.d.$_{50}$/0.5 Gm. of mouse lung. Although it was somewhat difficult to infect mice with suspensions of placental tissue from infected ewes, intranasal inoculation of about 10^5 e.l.d.$_{50}$ of yolk sac or mouse lung virus in a volume of 0.05 ml. into weanling mice resulted in the death of half of these animals. Of 81 mice given fatal doses of virus in the course of several titrations, deaths were distributed from the first (1%) to the ninth (2%) day, with a peak (26%) on the fourth day following inoculation. Lungs from these mice usually showed almost complete consolidation, and smears of the affected tisue showed elementary bodies.

The M virus was readily passed through four transfers in mouse lung. Parallel transfers of normal mouse lung gave no evidence of a PL group virus.

Intracerebral Inoculation.—After shallow intracerebral inoculation of large doses (10^6 to 10^7 e.l.d.$_{50}$) of M virus, most mice remained well. However, a few appeared ill for a week or two, and occasionally 1 died between the first and the seventh day following inoculation. Virus was demonstrated in meningeal smears of some of these. After 30 days all surviving mice appeared normal. When they were killed, though no gross lesions were apparent in the brain, virus was usually demonstrable by meningeal smear or yolk sac culture.

Intraperitoneal Inoculation.—When large doses (10^8 e.l.d.$_{50}$) of yolk sac virus were given to mice intraperitoneally, some deaths occurred within the first week and elementary bodies were found in smears of peritoneal fluid or organs. After inoculation of moderate doses, mice did not appear ill, none died, and elementary bodies were not readily demonstrated.

Intravenous Inoculation.—As will be described later, intravenous inoculation of 0.5 ml. of a fresh suspension of highly infected yolk sac produced death from toxicity within 48 hours, but a varying proportion of mice survived. Among these, deaths due to infection began to occur on the third day.

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A fresh yolk sac preparation diluted 1:10 failed to kill any mice within 48 hours, but killed 5 out of 10 from infection between the third and the ninth day.

PATHOGENICITY FOR GUINEA PIGS

Intraperitoneal Inoculation of Nonpregnant Animals.—Following an intraperitoneal inoculation of 0.5 ml. of a 1:10 dilution of sheep placental membrane and cotyledons into 3 adult guinea pigs, no temperature rises above 103 F. or other signs of disease were observed. All of these animals were later found unreactive to a challenge dose which produced fever in controls, indicating that they had undergone subclinical infections (table 2).

Fig. 1—Necrotic foci were present in the liver of a guinea pig four days after inoculation of $10^{5.5}$ e.l.d.$_{50}$ of M virus.

When yolk sac material with a virus content of about $10^{5.4}$ e.l.d.$_{50}$ was given, temperatures of over 103 F. occurred on the second or third day, or both, in 11 out of 12 animals. Smears of peritoneal fluid at this time showed no elementary bodies. While no signs other than fever were noticed in some inoculated animals, others showed inappetence, emaciation, and rough coat. Temperatures were usually normal again on and after the fourth day until the end of the two-week observation period when all guinea pigs appeared healthy.

Four days after inoculation with pyrogenic doses of M virus, 3 guinea pigs were killed and examined. No change in the peritoneal fluids was noticed and there was no fibrin deposit on the peritoneal surfaces. The livers were friable and numerous minute lesions which appeared grossly to be necrotic foci ranging from pinpoint to 2 mm. in size were distributed over their entire surfaces (fig. 1). Spleens were somewhat enlarged and congested. Kidneys were normal or slightly congested. Scrotal reactions were not found. Microscopically, the livers showed a few small foci of cellular infiltration and of karyorrhexis, fatty degeneration, and necrosis of the hepatic cells. Vacuoles, presumably fat, were quite generally distributed in the parenchymal cells. No lesions were observed in spleen or kidneys. Elementary bodies were not found in smears of peritoneal fluid or organs, but virus was readily isolated by yolk sac culture. Cultures failed to show bacterial growth.

TABLE 2—Antibody Response of Guinea Pigs to Intraperitoneal Inoculation of M Virus

Guinea pig No.	Date inoculated	Material	e.l.d.$_{50}$	Dilution	Maximum temperature* (F.)	Date blood obtained	Complement-fixation titer: Psittacosis (6 BC strain) 1:300	Complement-fixation titer: Q fever
1	.....	.....	.....	.....	102.0	3/29	0	0
	3/29	Sheep Placenta #1	$10^{4.6}$	1:10	102.2	4/22	1,024	0
	4/22	Yolk sac #1	$10^{5.5}$	1:2,000	100.5	5/9	1,024	0
2	.....	.....	.....	.....	100.8	3/29	0	0
	3/29	Sheep Placenta #1	$10^{4.6}$	1:10	101.4	4/22	64	0
	4/22	Yolk sac #1	$10^{5.5}$	1:2,000	101.1	5/9	1,024	0
3	.....	.....	.....	.....	100.6	3/29	0	0
	3/29	Sheep Placenta #1	$10^{4.6}$	1:10	101.0	4/22	192	0
	4/22	Yolk sac #1	$10^{5.5}$	1:2,000	100.4	5/9	1,024	0
4	.....	.....	.....	.....	99.3	4/22	0	..
	4/22	Yolk sac #1	$10^{5.5}$	1:2,000	103.7	5/9	1,024	..
5	.....	.....	.....	.....	99.2	4/22	0	..
	4/22	Yolk sac #1	$10^{5.5}$	1:2,000	105.0	5/9	1,024	..

* First figure for each is the preinoculation temperature. The following figures are the maximum temperatures shown on the second or third day postinoculation.

An intraperitoneal inoculum containing 10^8 e.l.d.$_{50}$ of fresh yolk sac virus was given to each of 2 guinea pigs. One of these remained afebrile and died on the second day. Death undoubtedly was due to toxin. The other died on the sixth day. Elementary bodies were not found in smears of peritoneal fluid or organs of these animals.

Intraperitoneal Inoculation of Pregnant Animals.—Four pregnant guinea pigs were each given $10^{5.3}$ e.l.d.$_{50}$ of yolk sac virus by the intraperitoneal route one month before the end of gestation. Three had elevated temperatures and 1 of these died on the eighth day. At necropsy this animal showed acute M virus infection. Smears of the cotyledons showed no elementary bodies.

The 3 remaining animals were killed near term. Grossly, all sows and fetuses appeared normal. Elementary bodies were found in smears of cotyledons and amniotic fluid obtained from 2 animals, and in smears of peritoneal fluid and uterine exudate from 1. They were not seen in smears of liver, spleen, kidney, or lung.

ANTIGENIC CHARACTERISTICS

Toxin Production.—Toxin was demonstrated in fresh yolk sacs which showed high concentrations of virus in smears. The yolk sacs were ground, diluted in yolk, and centrifuged for ten minutes at 1,500 r.p.m. Mice weighing 10 to 20 Gm. were given 0.5-ml. intravenous inoculations of each dilution. One preparation, in which infected yolk sacs were diluted 1:40 in their own yolks, killed 7 of a group of 10 mice within 24 hours and all 10 mice within 48 hours. All preparations were free from bacteria. Control inoculums of yolk and normal yolk sacs caused no deaths.

Demonstration of Psittacosis-Lymphogranuloma Group Antigen.—Of 8 guinea pigs that were given varying doses of living M virus intraperitoneally, 7 showed a significant rise in complement-fixation titer with heated antigen prepared from psittacosis virus.* Details of some of these results are given (table 2). When 10 pregnant ewes were inoculated subcutaneously with $10^{5.5}$ e.l.d.$_{50}$ of yolk sac virus, 8 showed significant rises in antibody titer with heated psittacosis antigen. None showed a significant rise with Q fever antigen. After being stored for three months, paired serums from 2 of these animals were tested and showed significant rises to heated EAE antigen.** Serums from 4 naturally infected ewes showed significant titers with both antigens (table 3).

In order to compare the group antigens of the two ovine strains, a heated complement-fixing antigen was prepared in this laboratory from the M virus by Stamp's method.[20] Duplicate dilutions were made from negatively and positively reacting sheep serums. One series was tested for fixation of complement with M virus antigen while the other was tested with EAE virus antigen.† Each serum reacted in an identical manner to both antigens.

Demonstration of Immunity.—The production of immunity in 3 guinea pigs 23 days after inoculation with living M virus was demonstrated by their failure to undergo a febrile response to the challenge dose which was pyrogenic for animals not previously infected (table 2).

Demonstration of Cross Immunity.—In order to determine whether inoculation of EAE virus would also protect against a pyrogenic dose of M virus, guinea pigs were given two inoculations of the Stamp strain at an interval of 16 days (table 4). Twenty-nine days later these animals and 4 controls were each given a challenge yolk sac dose of $10^{5.5}$ e.l.d.$_{50}$ of M virus. On the second and third days following challenge maximum temperatures for the previously inoculated guinea pigs were below 103 F., while maximum temperatures of controls were above this value.

COMPARISON WITH OTHER VIRUSES

For purposes of comparison, some pathogenicity tests were carried out with the virus of sporadic bovine encephalomyelitis, and with the Stamp strain.

Sporadic Bovine Encephalomyelitis Virus.—The growth of the sporadic bovine encephalomyelitis (SBE) virus in eggs and its pathogenicity for mice on intranasal, intraperitoneal, and intracerebral inoculation were found to be the same as that of

* Psittacosis and Q fever titers (table 2, 3) were kindly determined for us by Dr. David Lackman of the National Microbiological Institute, Rocky Mountain Laboratory, Hamilton, Mont.

** The EAE titers (table 3) were kindly determined for us by Dr. J. A. A. Watt of the East of Scotland College of Agriculture, Edinburgh.

† The EAE virus antigen was supplied by Dr. J. T. Stamp, Veterinary Investigation Center Laboratory, Edinburgh, Scotland.

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the M virus as far as these were studied. However, when 150- to 200-Gm. guinea pigs were inoculated intraperitoneally with material from infected calves, fever occurred later and was more persistent than after inoculation of M virus into adult guinea pigs. Fibrinous peritonitis, which was produced by the SBE agent, was not observed in M virus infection. Also, unlike M virus infection of nonpregnant animals, elementary bodies were found in the peritoneal fluid of some guinea pigs.

TABLE 3—Complement-Fixation Titers of Sheep Serums for PL-Group Antigen

Sheep	Remarks	Psittacosis titer	EAE Virus* titer
EXPERIMENTAL INFECTION			
1	Preinoculation	24	0
	22 days postinoculation	256	16
2	Preinoculation	16	0
	22 days postinoculation	256	64
NATURAL INFECTION			
3	Aborted three days before	128	1,024
4	Aborted a few days before	64	1,024
5	Aborted eight days before	1,024	256
6	Aborted five days before	1,024	16

* EAE = enzootic abortion of ewes.

The Stamp Strain.—In its growth in the yolk sacs of embroynating eggs and in the pathologic changes produced there, the Stamp strain resembled the M virus. Its behavior after allantoic inoculation was also observed to be similar. In its pathogeneity for mice, it was also found to be essentially the same as the M virus.

Symptoms observed in adult guinea pigs inoculated intraperitoneally with $10^{5.5}$ e.l.d.$_{50}$ of either M virus or Stamp strain were indistinguishable. As with M virus, when these animals were killed four days after inoculation of the Stamp strain, 2 of 4 showed numerous minute necrotic foci in the liver and slightly enlarged spleens.

DISCUSSION

The M virus was shown to possess the characteristics which establish it as a member of the PL group. While it was shown to contain the PL group antigen, it did not contain the complement-fixing antigen of the Q fever agent. In contrast to the Rickettsiaceae and in common with most other members of the PL group, M virus was inhibited by a sulfonamide and by penicillin and was unaffected by streptomycin or by *p*-aminobenzoic acid.

These properties served to rule out the possibility that the agent was *Coxiella burnetii*, which may also occur in fetal membranes and fluids of sheep and has been considered a possible etiologic agent of abortion.[3,15]

It remained, therefore, to determine whether the M virus was identical with one or more of the known members of the PL group or was a new member. It was found to be less virulent for mice on intracerebral or intraperitoneal inoculation and for chicken embryos than psittacosis strains of avian origin. Also, unlike them, M virus was sensitive to sulfathiazole. However, it resembled PL strains of mammalian origin,

TABLE 4—Cross Immunity Tests in Guinea Pigs

Guinea pig No.	Date inoculated	Strain	e.l.d.$_{50}$ or dilution	Material	Maximum temperature (F.)
1					99.7
	7/23	Stamp	$10^{7.1}$	Yolk sac #2	103.6
	8/8	Stamp	1:4	Mouse lung #1	103.1
	9/5	M	$10^{5.5}$	Yolk sac #3	102.4
2					99.8
	7/23	Stamp	$10^{7.1}$	Yolk sac #2	104.1
	8/8	Stamp	1:4	Mouse lung #1	103.5
	9/5	M	$10^{5.5}$	Yolk sac #1	102.3
3					101.1
	7/23	Stamp	$10^{7.1}$	Yolk sac #2	104.8
	8/8	Stamp	1:4	Mouse lung #1	102.7
	9/5	M	$10^{5.5}$	Yolk sac #3	102.8
4					99.5
	7/23	Stamp	$10^{7.1}$	Yolk sac #2	103.6
	8/8	Stamp	1:4	Mouse lung #1	102.8
	9/5	M	$10^{5.5}$	Yolk sac #3	102.0
5					101.5
	9/5	M	$10^{5.5}$	Yolk sac #3	104.9
6					101.8
	9/5	M	$10^{5.5}$	Yolk sac #3	103.2
7					101.2
	9/5	M	$10^{5.5}$	Yolk sac #3	105.0
8					101.0
	9/5	M	$10^{5.5}$	Yolk sac #3	105.5

such as the SBE virus,[21] York and Baker's enteric virus of calves,[22] McKercher's virus of sheep pneumonia,[9] and several others.

The similarity between EAE as it occurs in Scotland and the disease produced in sheep by the M virus suggests that they are caused by identical viruses. The appearance of the M virus in smears from aborted sheep placentas[19] and its distribution in the fetal organs and membranes were as described[8] for the EAE virus. The stability of the two agents under various temperatures of storage,[12,19] inhibition by antibiotic and chemotherapeutic drugs,[2] and toxin production[10] were the same as far as studied. Growth in embryonating eggs was observed to be the same for both strains.

The results of the inoculation of laboratory animals with M virus by any route vary with dosage. Inoculation of mice with either M virus or Stamp strain by the intraperitoneal, intracerebral, or intravenous route was not lethal except when very large doses (10^6–10^8 e.ld.$_{50}$) were used. If the effect of differences in dosage is taken into account, most of these findings appear to be compatible with those recorded in the literature for EAE virus.[4, 10, 12, 19]

The results of intraperitoneal inoculation of M virus into guinea pigs vary, according to dose, from subclinical disease to clinical disease and, with massive doses, to death. In guinea pigs inoculated intraperitoneally with placental suspension, Stamp *et al.*[19] reported a temperature rise of 1 to 2 F. with no pathologic changes. As already mentioned, Giroud *et al.*[4] report more pronounced effects in animals similarly inoculated with uterine exudate from ewes. It appears probable that these differences are largely due to variation in the amount of virus in the inoculum.

The production of placental infection by intraperitoneal inoculation into pregnant guinea pigs as observed in this laboratory for M virus, was reported by Stamp *et al.*[19] for EAE virus.

When tested for complement fixation with heated antigen prepared from the EAE virus, serums from ewes which had been either naturally or artificially infected with M virus reacted in a manner considered diagnostic of enzootic abortion of ewes.[18] Although they share a common heat-stable component, PL group antigens prepared from different members of the group apparently do not always act in exactly the same manner.[1, 13] The identical behavior observed in complement-fixation tests with heated antigens prepared from M virus and from EAE virus is the result that would be expected if the viruses were the same.

The production of resistance to M virus infection in guinea pigs inoculated with Stamp strain indicates a close relationship between the two agents. Also, Stamp[18] has observed that M virus possesses the thermolabile specific complement-fixing antigen described by Monsur and Barwell[14] as characteristic of the EAE virus.

Thus, these viruses have been shown to be indistinguishable in their pathogenicity for animals, in their antibiotic sensitivity, and in their antigenic composition so far as studied. It therefore appears reasonably certain that M virus and EAE virus are the same.

The relationship of this virus to some other mammalian viruses of the PL group appears uncertain. It is not clear whether the differences observed may be due to differences in the technique used in testing the viruses, whether they may be present in only certain strains among those isolated from a single clinical syndrome, whether they may represent readily reversible adaptations to repeated passage in the same host animal, or whether they are relatively permanent characteristics present alike in all strains isolated from each single clinical disease. Until further evidence is available, the possibility exists that two or more of these viruses are the same. A similar opinion regarding some of these closely related viruses has been expressed by Kawakami *et al.*[7]

SUMMARY

A virus considered to be in the psittacosis-lymphogranuloma venereum (PL) group was isolated from sheep in Montana which showed signs closely resembling those of enzootic abortion of ewes as described by Stamp. This agent exhibited an affinity for yolk sac tissue of embryonating eggs, mouse lung, and placental tissues of ewes and guinea pigs. It gave rise to latent infections in mice and in pregnant ewes and guinea pigs. Moderate doses produced little or no clinical disease in mice by the intraperitoneal, intracerebral, or intravenous route, but were lethal on intranasal administration. Intraperitoneal inoculation of moderate doses into guinea pigs produced a typical clinical and pathologic syndrome which resulted in recovery and resistance to reinfection.

Cross immunity between this virus and the Stamp strain was demonstrated in guinea pigs. As its characteristics corresponded closely to those observed in this laboratory or reported by others for the virus of enzootic abortion of ewes, it was identified as a strain of that virus. However, its presence in this country focuses interest on the question of its relationship to other PL group viruses found here, such as the virus of sporadic bovine encephalomyelitis, York and Baker's enteric virus of calves, and McKercher's virus of sheep pneumonia.

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Serologic Investigation of Pseudotuberculosis in Sheep. I. Agglutination Test

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PSEUDOTUBERCULOSIS or caseous lymphadenitis of sheep is a common disease in Sudan, particularly in the western part of the country. Since the disease is seldom diagnosed in the living animal, most of the information on the frequency of the disease comes from meat inspection statistics.

Maddy,[5] trying to show the economic importance of the disease, indicated that in the United States in 1952, 12,056 of 68,965 sheep were condemned because of caseous lymphadenitis. He also stated that this disease had not received adequate attention in regard to diagnosis or prevention.

It is generally accepted that no real approach to the control of this disease could be carried out without an efficient diagnostic method which could be applied to the living animal. Cassamagnaghi [3] reported on the value of pseudotuberculin C as a diagnostic agent when injected, like tuberculin, into the caudal fold. He claimed 100 per cent success.

Carne [2] reported that a critical trial in Australia of various allergic tests showed that none had a degree of efficiency to warrant its adoption.

Cameron and McOmie [1] did some work with the agglutination test and also prepared a pseudotuberculin for the intradermal test, but little use has been made of their findings.

The high incidence of pseudotuberculosis in Sudan stimulated research on that disease. This paper presents an evaluation of the agglutination test in the diagnosis of caseous lymphadenitis in sheep.

MATERIALS AND METHODS

Preparation of the Antigen.—A large number of *Corynebacterium ovis* strains were collected from Khartoum and Elobied abattoirs. After cultivation on agar, each strain was separately emulsified in normal saline solution and the rate of its precipitation recorded. Most of the strains were found to precipitate immediately, leaving the supernatant fluid clear within a few minutes. Strain 58 was found to stay in suspension for a few hours, and even after standing overnight in a refrigerator some turbidity showed in the supernatant fluid. This strain was chosen for the preparation of the antigen.

Approximately 5 cc. of a 48-hour serum broth culture of the strain was added to each Roux bottle of serum agar. The bottle was rocked gently so that a uniform distribution of the seed was obtained. The seeded bottles were inverted after half an hour and incubated for 48 hours at 37 C. After incubation, each bottle was examined macroscopically. Bottles with contaminated contents as well as those in which the medium had slipped were discarded. Condensed water and supernatant inoculum were poured off, and 10 cc. of sterile saline solution was added to each bottle. The bottle was agitated until the growth became suspended; the suspension in each bottle was then removed to a sterile container.

A Gram-stained smear preparation was made from each suspension and examined microscopically for purity; if found satisfactory for antigen production, the suspension was filtered through sterile glass wool and centrifuged at a high speed. The packed organisms were then washed three times in saline solution. This constituted the stock organisms for the preparation of the antigen.

Since *C. ovis* is prone to clumping, a uniform bacterial suspension for the agglutination test was achieved by using different salt concentrations (0.85 to 15%) and different pH values (6.0 to 8.0). It was found that the best diluent was a 5 per cent salt solution containing 10 to 20 per cent normal rabbit serum which had been heated for 30 minutes at 56 C. with the pH adjusted to 6.8. After the addition of the diluent, the organisms were shaken with glass beads for half an hour and then allowed to stand overnight in a refrigerator. The sediment was discarded; the supernatant fluid constituted the stock antigen. For the tube test, the antigen was standardized to Brown's tube No. 3 and for slide test, to ten times tube No. 5.

Titration of the antigen for both tube and slide tests was carried out according to Kolmer's method [4] against rabbit immune and negative serums.

For the tube test, Kolmer's technique [4] was adopted. For the slide test, a drop of concentrated antigen was mixed with an equal amount of fresh

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serum on a slide, the slide was rocked back and forth 15 to 20 times, and the mixture examined for agglutination. Various degrees and speeds of agglutination were noticed. The results were recorded as complete, incomplete, and negative. In each batch of serums examined a known positive and a negative serum were included.

EXPERIMENTAL RESULTS

Specificity of the Agglutination Test.—The slide agglutination test was carried out using *C. ovis* and *Brucella abortus* antigens on serums from 20 sheep showing lesions of pseudotuberculosis, from 20 cattle with brucellosis, and from 20 sheep with Staphylococcus infection in the prescapular lymph nodes. The results are shown (table 1).

TABLE 1—Specificity of the Slide Agglutination Test

No. of animals	Type of infection	No. positive	
		C. ovis antigen	*Br. abortus* antigen
20 sheep	*Corynebacterium ovis*	18	0
20 cattle	*Brucella abortus*	0	20
20 sheep	Staphylococcus	2	0

Of 20 sheep with pseudotuberculosis lesions, 18 reacted positively to *C. ovis* antigen; all were negative to brucella antigen. None of 20 cattle with brucellosis reacted to the *C. ovis* antigen. Of 20 sheep with staphylococcal infection, 2 reacted positively to *C. ovis* antigen and none reacted to the brucella antigen.

Agglutination Tests on Sheep Artificially Infected with Corynebacterium ovis.—On December, 1957, 12 lambs were brought to the Research Division. The serums from 5 of these lambs gave incomplete agglutination in the slide test; these animals were rejected. Of the 7 lambs with negative reactions, 2 (54 and 59) were kept as controls and 5 (49, 50, 52, 55, and 57) were each inoculated subcutaneously with 2 ml. of 48-hour broth culture of *C. ovis*. Serums were collected from these animals at different intervals for the agglutination test (table 2). At necropsy 40 weeks postinoculation, no lesions were detected in the 2 control sheep. Local infections were found in the prescapular lymph nodes of all inoculated sheep; the lungs of sheep 52 and 57 were also affected.

This experiment showed that, as far as the tube agglutination test is concerned, a titer of 1:32 is considered as negative and 1:64 as doubtful. Taking these titers in consideration, table 2 shows that seven weeks after infection 3 animals reacted doubtfully to the tube test but only 1 to the slide test; 40 weeks after infection, 2 animals reacted doubtfully to the tube test but none to the slide test. Nevertheless, the results obtained showed a close agreement between the two tests.

The Slide Agglutination Test on Sheep Serums for Detection of Natural Infection.—Serums were collected from 264 sheep showing abscesses in the prescapular lymph nodes and from 519 sheep with no macroscopic lesions. An agar culture was made from blood of each sheep with lesions and the agglutination test was carried out on its corresponding serum (table 3).

Of 264 sheep showing lesions during meat inspection, 197 were infected with *C. ovis*, 43 with Staphylococcus, and in 24 animals no culture could be obtained due to advanced caseation or calcification or both (table 3). Of the 197 sheep with psuedotuberculosis, 188 were positive to the test, while in all the animals with Staphylococcus the test was negative. Of 519 sheep with no lesions, 53 gave positive reactions to the test.

DISCUSSION

The strong tendency of various strains of *C. ovis* to clump makes it difficult to prepare a uniform bacterial suspension suitable for serologic work. The first problem was to determine whether or not a *C. ovis* strain could be found which would yield a

TABLE 2—Results of Agglutination Tests on Artificially Infected Sheep

No. Sheep	4 Weeks		7 Weeks		18 Weeks		40 Weeks	
	Slide	Tube	Slide	Tube	Slide	Tube	Slide	Tube
49	incomplete	1:64	complete	1:256	complete	1:256	complete	1:64
50	complete	1:128	complete	1:256	complete	1:256	complete	1:128
52	complete	1:128	complete	1:512	complete	1:1,024	complete	1:256
55	complete	1:64	complete	1:128	complete	1:128	complete	1:64
57	complete	1:64	complete	1:256	complete	1:256	complete	1:256
54	negative	1:32	negative	1:32	negative	1:32	negative	1:32
59	negative	1:16	negative	1:32	negative	1:32	negative	1:32

suspension suitable for the agglutination test. This was achieved by studying the clumping rate of many strains of *C. ovis*; strain 58 was chosen as suitable. The strain was tested in different salt concentrations and pH values. It was finally found that the best bacterial suspension for the agglutination test was obtained in 5 per cent salt solution containing 10 to 20 per cent heated normal rabbit serum with the pH adjusted to 6.8.

TABLE 3—The Results of Culture and Slide Agglutination Tests on Sheep

No. of sheep	Type of infection	No. positive (slide agglutination)
264 with lesions	197 *C. ovis*	188
	43 Staphylococcus	0
	24 negative	6
519 without lesions	.....	53

The antigen so prepared was found to give specific agglutination with known immune serums, and evidence of its reliability was demonstrated by testing serums of naturally infected sheep.

Table 2 indicates that as far as the tube agglutination test is concerned a titer of 1:32 is negative and 1:64 doubtful; it also compares the value of the slide test with the tube test. The results (table 2) showed close agreement between the tube and slide agglutination tests, but the slide test is preferred for its practical advantages of economy and speed.

The third experiment demonstrated the efficiency of the slide agglutination test; of 783 sheep tested, 247 were positive but 59 of them had no detectable infection. There was no reaction in 9 sheep with detectable infection. It is inferred that 256 of the 783 sheep were infected with *C. ovis;* if so, the average failure rate of the slide agglutination test is 3.5 per cent.

It is concluded that the present investigation has shown that the slide agglutination test can be regarded as a reliable and practical serologic method in the diagnosis of pseudotuberculosis in sheep.

SUMMARY

1) This study describes the use of the agglutination test as a method for diagnosing pseudotuberculosis in sheep.

2) A bacterial suspension of *Corynebacterium ovis,* strain 58, yielded the most potent agglutination antigen in a 5 per cent salt solution containing 10 to 20 per cent heated normal rabbit serum with the pH adjusted to 6.8.

3) The test is specific in the diagnosis of caseous lymphadenitis; in the tube test a titer above 1:64 is significant.

4) It is concluded that the slide agglutination test is a reliable and practical method in the diagnosis of pseudotuberculosis and preferable to the tube test.

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Experimental Trichostrongylosis *(axei)* in Lambs, with a Discussion of Recent Research on this Disease in Ruminants

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THE SMALL trichostrongylid nematode, *Trichostrongylus axei*, occurs commonly in the stomach of equine animals and the abomasum of various domestic and wild ruminants. A number of reports in the literature of disease in ruminants on pasture, thought to be caused primarily by this parasite, have been summarized to a large extent by Dikmans and Kates [2] and Doran.[3] Recently, extensive experimental work has been reported by Gibson,[6-11] Spedding,[16-18] Doran,[3] Leland *et al.*,[13,14] and the authors,[19] which has clarified the relationship of *T. axei* to disease in ruminants. It is now clear that cattle and sheep, when invaded with large numbers of *T. axei*, develop a severe form of parasitic gastritis accompanied by a variable duodenitis, as well as other clinical and physiological effects. Eveleth and Bolin [5] have also reported acute cases of this disease in elk.

In addition to these studies on ruminants, Drudge *et al.*[4] and Leland and Drudge [12] have shown that the domestic rabbit and certain other small laboratory animals are susceptible to infection with this parasite. It is also of interest that Rohrbacher [15] has employed the *T. axei*-rabbit, host-parasite system to test the effect of different feeds on development of the parasite.

The purpose of this paper is to report the results of our work in more detail than was previously reported in part in abstract,[19] and to discuss some aspects of recent developments on trichostrongylosis *(axei)* of domestic ruminants.

EXPERIMENTAL PROCEDURE

Twelve purebred Shropshire lambs, 2 to 3 months of age, raised parasite-free except for insignificant infections of coccidia and *Strongyloides papillosus*, were used in this experiment. Six lambs were inoculated with *T. axei* by oral administration of single doses of 50,000 to 500,000 infective larvae (table 1). Each inoculated lamb was matched with an uninoculated lamb of comparable weight; the average weight was 34 lb. Lambs of the inoculated and control groups were maintained in separate concrete-floored pens without bedding throughout the experiment. Two pens were available for each group, which was moved to its alternate clean pen daily to prevent extraneous infection. All lambs were rack-fed good grade alfalfa hay in excess throughout the experiment.

The strain of *T. axei* employed was the same as that used by Doran.[3] It was originally obtained from cattle and established in a parasite-free lamb, as described by Dikmans and Kates.[2] Originally a light infection was established in this lamb, which consisted of about 84 per cent *T. axei* and about 16 per cent *Trichostrongylus colubriformis*.[2] However, upon subsequent passages through several otherwise parasite-free lambs previous to the start of this experiment, the proportion of *T. colubriformis* decreased to less than 1 per cent. This percentage was determined by counts of worms recovered at necropsy from lambs used as sources of feces for culture and from the infected lambs in this experiment. Consequently, for all practical purposes the lambs in this experiment were considered to be infected only with *T. axei*.

Daily observations were made on the lambs for appearance and intensity of signs of infection. At weekly intervals, weights were taken as well as blood samples for hemoglobin and hematocrit determinations. When the infections became patent, the number of worm eggs per gram a feces was ascertained semiweekly by the direct centrifugal flotation-brine method. Since the infected lambs rapidly developed acute signs of the disease, they were necropsied on postinoculation days 36 to 42 (table 1) with the exception of lamb 5. At necropsy, ligatures were tied at the juncture of the omasum and abomasum, at the pyloric valve, at the junction of each of the first 3 ft. of duodenum, and at the ileocecal valve. The gastrointestinal tract was then removed and small pieces of abomasal and intestinal tissues were excised and fixed in Bouin's fluid for later histologic study. The digestive tract was then opened and the worms in the various sections were recovered by thorough washing and by rubbing the surface of the mucosa manually.

RESULTS

Clinical Effects and Worm-Egg Counts.—The general clinical effects of the infec-

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tions on the lambs were much the same regardless of the number of larvae administered, except that lamb 6, which had been given the largest number of larvae, was less affected than the others. The first clinical signs were observed during the second week of the experiment and included depression of feed and water consumption, and reduction in rate of gain. Diarrhea began at the end of the second week after infection in lamb 3. It began at the end of the third week in lambs 1, 2, 4, and 5, and lasted for varying periods (table 1 and fig. 1A). The feces became light brown and the diarrhea was severe, watery, and was sometimes present for several days. In some instances, periods of diarrhea were interspersed with intervals during which soft unformed feces were passed. Lamb 6 never developed diarrhea but passed soft or mushy feces intermittently on 17 of the 36 days following inoculation. The fact that the first signs of infection occurred during the second week of the experiment indicated that immature worms were capable of producing pathogenic effects when present in suffieiently large numbers. Lamb 5 died on postinoculation day 30 and lambs 1 to 4 become moribund and were killed *in extremis* on 36 to 42 days.

At the time of death, the average weight of lambs 1 to 5, inclusive, was about 25 lb. less than that of their comparable controls. All the infected lambs lost weight during the course of the experiment, and their terminal weights varied from 2.0 to 15.5 lb. less than their initial weights (fig. 2C). Lamb 5, which died 30 days after inoculation, weighed 39.5 lb. at the beginning and only 24.0 lb. at death, a loss of 15.5 lb. Lamb 6, the largest of the group, lost weight more rapidly than the others during the second week only. However, in the end it was the least affected, although it lost 2 lb. during the experiment, whereas its control gained 12 lb. It is probable, therefore, that before patency this lamb retained more immature worms than the other lambs, and that most of these were spontaneously eliminated before reaching maturity, as shown by the fecal egg counts (fig. 2A) and the terminal worm counts (table 1).

Significant differences developed in hemoglobin and hematocrit values between the infected and control lambs. Only hemoglobin values are given in figure 2B as the hematocrits showed the same trend. Lambs 1 to 5, inclusive, developed a relative polycythemia by the end of the fourth week after inoculation. This trend continued to the end of the experiment. The final average hemoglobin values for these lambs was 16.4 per cent, and the final average hematocrit reading was 20.0 per cent higher than that of the controls. When compared with their own initial values, these differences were even more marked, amounting to 20 to 25 per cent, respectively. Lamb 6, however, showed the opposite trend. Its final hemoglobin and hematocrit readings were 19 per cent and 18 per cent, respectively, lower than those of its control. These same differences also

TABLE 1—Data on Experimental Infections of Lambs with Trichostrongylus axei

Lamb No.	Larvae administered (thousands)	Period from infection to necropsy* (days)	Period of acute diarrhea (days)	Worms recovered at necropsy			
				Abomasum (No.)	Small intestine (No.)	Total (No.)	Larvae administered (%)
1	50	42	7	45,800	62	45,862	91.7
2	75	42	8	36,750	1,163	37,913	50.5
3	100	36	5	31,250	356	31,606	31.6
4	200	36	2	20,725	1,023	21,748	10.8
5	300	30	7	10,925	2,960	13,885	4.6
6	500	36	0	4,875	845	5,720	1.1

* Lambs 1 to 4, inclusive, killed in extremis; lamb 5 died; lamb 6 was in poor condition when killed but not *in extremis*.

Data on worm egg counts, hemoglobin, and body weight are summarized (fig. 2). Eggs of *T. axei* first appeared in the feces three weeks after inoculation, except in those of lamb 6, and rapidly increased in numbers in the succeeding weeks (fig. 2A). Although there was considerable variation in the fecal egg counts, lambs 1 and 2, which were given the smallest doses of larvae, had the highest egg counts. The largest single egg count of 32,000 eggs per gram (e.p.g.) was obtained from lamb 2, and the maximum egg count recorded from lamb 6 was only 84 e.p.g.

were recorded between the final and initial values of these readings.

Postmortem Observations and Worms Recovered.—At necropsy, lamb 6 was in somewhat better condition than the other 5 lambs. The fact that anorexia was a common sign of infection was confirmed by the absence of significant quantities of food material in the alimentary tracts of lambs 1 to 5, inclusive, and only a small quantity in lamb 6. Typically, the kidneys and livers were soft and friable, with the livers dark in color; the lungs showed some congestion. Hemoconcentration was grossly evidenced in lambs 1 to 5 by the abnormally dark red color of the blood and its unusually thick consistency. The abomasum and duodenum of these lambs were enlarged, edematous, and hyperemic. However, the extent and severity of the enteritis was variable in the duodenum, occurring from 1 to 12 inches from the pyloric valve. The hyperemia and edema were particularly marked internally in the areas adjacent to the pyloric valve. Usually, the contents of the abomasum and small intestine consisted of a catarrhal exudate containing much cellular debris. In most cases, there appeared to be considerable erosion of the mucosa of the pyloric region of the abomasum and duodenum. No other significant gross lesions were noticed.

The number of worms recovered from the 6 infected lambs was roughly inversely proportional to the number of larvae administered (table 1). Only adult worms were recovered. It appeared that as the larval dosage rates increased above 50,000, the numbers of worms that reached maturity and lived for significant periods of time sharply declined. Because *T. axei* is primarily an abomasal nematode, the bulk of the worms was recovered from this organ. However, an average of 4 per cent of the total populations of the 6 lambs was recovered from the small intestines, and the majority of these were from the duodenum. Probably, the relatively small numbers of *T. axei* recovered from the small intestines were in the process of being spontaneously eliminated from the hosts, except for possibly a few that had become lodged in the mucosa of the duodenum adjacent to the pyloric valve.

Histopathology.—The obvious histologic changes appeared to be localized in the abomasum and to a variable extent in the duodenum (fig. 3A and B), and were similar to those reported by Doran [3] in the abomasum of calves with acute infections of the same strain of *T. axei.* Sections of the affected areas of the abomasum and duodenum showed much erosion of the superficial mucosa, edema in the submucosa and muscle layers, variable small ulcerated areas in the mucosa, hyperemia, and considerable cellular infiltration, mainly lymphocytic, in the mucosa.

Athough the vast majority of the worms were in the lumen of the abomasum at necropsy and to a lesser extent in the lumen of the duodenum and small intestine, small numbers of worms were observed in the mucosa, mainly superficially located in the gastric mucosa (fig. 3A) or, more rarely, in the duodenal mucosa (fig. 3B). Rarely, the anterior end of a worm was seen deep in the mucosa adjacent to the muscularis. Because of the presence of a few *T. colubriformis* in these infections, the writers cannot be certain that those few worms observed in duodenal tissue were *T. axei,* but there can be little doubt that the abnormal appearance of the duodenum was caused by the latter species. Sections of the small intestines other than the obviously affected areas of the duodenum, appeared normal except for slight erosion of the epithelial surface of the mucosa, which was probably secondary to the acute effects present in the abomasum and the immediate adjacent area of the duodenum.

DISCUSSION

No experimental work with *T. axei* had been reported prior to that of Gibson,[6] who employed a sheep strain of the parasite in sheep in this and succeeding studies. Subsequently, Gibson [7–11] and Spedding [16–18] in England, and workers in this country,[3, 4, 12, 13, 14, 19] carried out further experimental studies and demonstrated that this parasite had considerable potential for pathogenesis in domestic ruminants. These experimental results are largely complementary and confirmatory. The experimental infections, which have been carried out with cattle and sheep under different conditions as well as with strains of the parasite of different host and geographical origin, add to the value and comprehensiveness of the available information on this small but important nematode parasite.

The combined reports of Gibson,[6-11] Doran,[3] Drudge *et al.*,[4] and the writers [19] indicate that the natural ruminant hosts and one experimental host (rabbit) are highly susceptible to infection with various equine, bovine, and ovine strains of *T. axei*. The reports also indicate that there is no apparent marked difference in virulence in strains, regardless of host origin.

Convincing examples of naturally acquired acute trichostrongylosis caused wholly or in part by *T. axei* were reported by Andrews *et al.*,[1] Dikmans and Kates,[2] and others cited by these authors. The fatal cases of almost pure *T. axei* infection in elk reported by Eveleth and Bolin [5] are of special interest because they were acquired on pasture and because the infections consisted of immature worms. This is in agreement with the writers' report that clinical signs appeared while the worms were immature. Likewise, Andrews *et al. (loc. cit.)* reported an outbreak of a similar disease condition in a herd of Brahman cattle. More than 400,000 *T. axei* were recovered from a mature cow at necropsy, a number comparable to the worm recoveries reported by Doran [3] and Leland *et al.*[13,14] from fatal experimental infections of cattle. Therefore, there is substantial evidence that massive and sometimes fatal infections of *T. axei* in domestic ruminants, which were experimentally produced by Gibson,[6-11] Doran,[3] Leland *et al.*[13,14] and the writers,[19] also have occurred under natural pasture conditions.

The results reported by Gibson [6-11] working with a sheep strain of *T. axei* and the writers working with a bovine strain are similar, except for certain minor differences. Gibson *(loc. cit.)* produced infections in lambs by daily administration of 4,000 larvae over 15 to 30 days (total larvae given each lamb—60,000 to 120,000 in different experiments), whereas the writers used single doses of 50,000 to 500,000 larvae per lamb. The divided dose method of Gibson produced typical abomasitis, as well as other effects, and death in some cases. But these effects were usually delayed in comparison with the acute effects reported herein. Often the infections became chronic and partial recovery occurred, which was probably the result of development of partial resistance to infection. Furthermore, lambs that died several weeks or months after administration of the last dose of larvae, as well as lambs that were severely affected but survived, showed some anemia. Also, at necropsy some small abomasal ulcers of varying number and appearance were seen.

The fatal infections established by the writers with 50,000 to 300,000 larvae, given in single doses to lambs, were essentially more severe than those reported by Gibson. The increased severity was shown by the acute diarrhea, rapid loss in weight, almost complete refusal of feed and water, and the rapidity with which death occurred. Instead of anemia occurring in 5 of our infected lambs, as Gibson reported in some cases, the opposite occurred—a relative polycythemia, probably brought about by the rapid water loss from the alimentary tract and reduced fluid intake. However, lamb 6, less severely affected than the other 5 lambs, survived and showed terminal anemia, similar to some of the cases reported by Gibson. Probably because of the

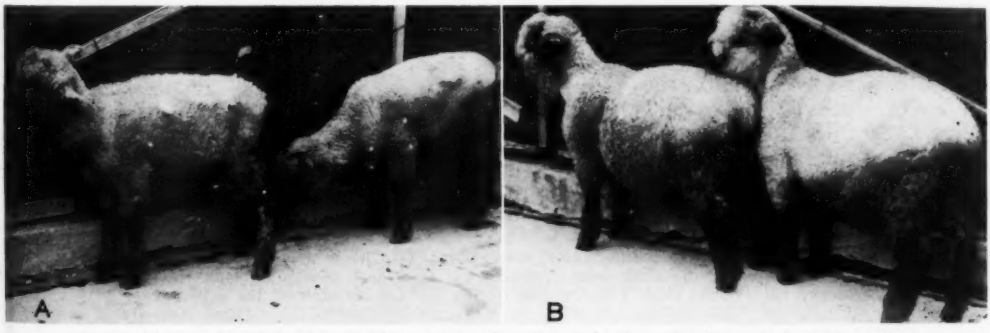

Fig. 1 (A)—Infected lambs 3 and 4, five weeks after infection, showing the typical debilitating effects of T. axei. Notice the fluid, light-colored feces on floor in right lower corner. (B) Two comparable uninfected control lambs.

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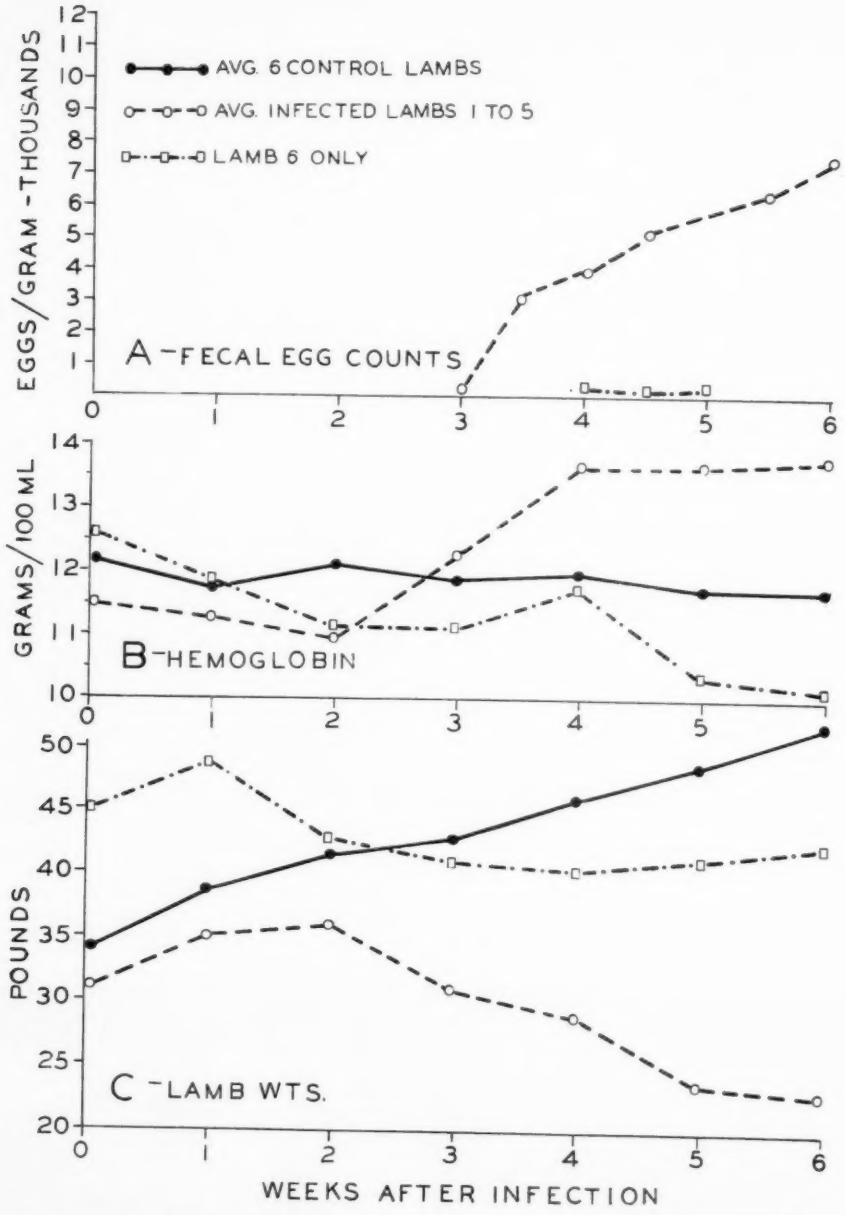

Fig. 2 (A)—Data on egg counts; (B) hemoglobin; (C) body weight. Data graphed for the infected lambs for the sixth week represent the final determinations made just prior to necropsy but exclude lamb 5 which died during the fourth week of the experiment.

early termination of our experiment compared with Gibson's, no gross abomasal lesions were found, but histologically the abomasal mucosa showed diffuse sloughing and minute ulcerations.

It would appear, therefore, that the trend in blood changes and gross abomasal pathologic changes in trichostrongylosis *(axei)* in lambs is partially governed by the degree of severity and duration of infection. In more or less chronic infections, the obvious signs are those normally following moderate anorexia and concomitant malnutrition, including a nutritional anemia, whereas in acute infections the refusal of feed and water, and acute diarrhea, results in rapid dehydration, hemoconcentration, weight loss, and death in many cases.

The results of two other recent experiments have important bearing on the pathogenesis of *T. axei* in ruminants. Spedding [18] showed that moderate, subclinical infections of *T. axei* in lambs, which caused only an 8 per cent decrease in appetite, caused a 10 per cent decrease in digestive efficiency. The recent work of Leland *et al.*[13] adds much valuable information on the changes in blood and plasma volumes and serum proteins in fatal and nonfatal infections in calves. They demonstrated that the direct and indirect effects of acute infections of *T. axei* on blood and plasma volume caused first the development of polycythemia hypovolemia which, if the animal survived, developed eventually into an oligocythemia hypovolemia. Also, the effect of the infections on total serum protein was one of prolonged hypoproteinemia which, when analyzed electrophoretically, consisted of a hypoalbuminemia and hypoglobulinemia, except for *alpha*-2-globulin, which either increased or maintained its normal level. Similar changes also probably occur in acute infections in sheep.

Enough information is now available that some assessment can be made of the relative pathogenicity of *T. axei* in cattle and sheep. The main differences in the effects of this parasite on these two hosts appear to depend upon the severity and duration of the infections and differences in host size, rather than intrinsic differences in host susceptibility. In acute infections in both hosts, almost total refusal of feed and water, acute diarrhea, emaciation, and general physical weakness are the main clinical signs, which may be accompanied by reduced blood volume and polycythemia. In animals with recovered or chronic cases, however, diarrhea is usually absent and feed and water intake may be reduced to a varying degree. Also, although a hypovolemia may be present (Leland *et al.*[13]), it is usually accompanied by an oligocythemia probably of nutritional origin (Gibson,[8] Leland *et al.*[13]). In addition to abomasitis, Doran [3] and Gibson [10] described "ringworm-like" lesions in the abomasum of calves and lambs, respectively, but the writers did not observe such lesions. Apparently, lesions of this type are associated with chronic or recovered cases, rather than rapidly developing acute infections. Histopathologic observations of various workers are much alike for the two hosts.

The work of Gibson,[6-11] Doran,[3] Leland *et al.*[13-14] and the writers is an adequate basis for estimating the minimum lethal dose of *T. axei* larvae for calves and lambs. Gibson gave lambs 60,000 to 120,000 larvae over periods of 15 to 30 days and some fatalities occurred. The writers produced a fatal infection in a lamb with a minimal single dose of 50,000 larvae. An intake, therefore, of 50,000 larvae over a short period by a susceptible lamb is within the lethal range, but it is also possible that lambs under some form of stress may die from an intake of less than 50,000. The combined reports of Doran [3] and Leland *et al.*[13-14] show that a single dose of 500,000 or more may be lethal for susceptible calves. On this basis, if the minimal fatal doses of larvae for lambs (50,000) and for calves (500,000) are taken as rough quantitative indicators of relative pathogenicity of *T. axei* in these two hosts, these figures give a ratio of 1 to 10 for minimal lethal numbers of infective larvae in susceptible lambs and calves. This lethal ratio of larvae in the two hosts is proportionate to the ratio of initial weights of lambs and calves used in these experiments. It would appear, therefore, that on a host-weight basis *T. axei* is equally pathogenic in sheep and cattle.

The conclusion above is supported by various data derived from reports of postmortem worm counts from sheep and cattle. Andrews *et al.*[1] reported over 400,000 *T. axei* from a naturally infected Brahman cow killed *in extremis*. Doran [3] recovered

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116,000 and 130,000 *T. axei,* respectively, from 2 fatally infected calves; 1 recovered calf harbored 167,000. Leland *et al.*[14] recovered 99,000 to 330,000 *T. axei* from 7 calves with fatal infections, and a maximum of 205,000 from calves with nonfatal infections. From lambs, however, the writers recovered approximately 13,000 to 45,000 *T. axei* from 5 with fatal cases, and Gibson *(loc. cit.)* in various papers reported recoveries of 21,900 to 57,200 from 14 lambs with fatal cases. Therefore, it appears that not only do cattle need to ingest more larvae of *T. axei* than sheep in order to develop acute trichostrongylosis, but also more *T. axei* may be retained in cattle than in sheep when death ensues from this disease.

It is still not clear, however, how this small unarmed nematode produces its injurious effects in the abomasal-duodenal region of the alimentary tract. That some mechanical irriation is produced is evident from the histologic observations of Doran,[3] the writers, and others, but it does not seem possible that this is severe enough to explain the total adverse effects of the parasite. Possibly the immature and adult *T. axei* may secrete or excrete substances that are yet to be isolated and identified. These substances may contribute to the localized abomasal and duodenal irritation, and the ensuing ingestive, digestive, and absorptive malfunction. The direct effects of the parasites in chronic and acute infections are certainly greatly enhanced and intensified by the secondary adverse effects on appetite and water intake and, as aptly expressed by Leland *et al.*,[13] "are thus supplemented, and sometimes overshadowed, by the ill effects of malnutrition and negative water balance."

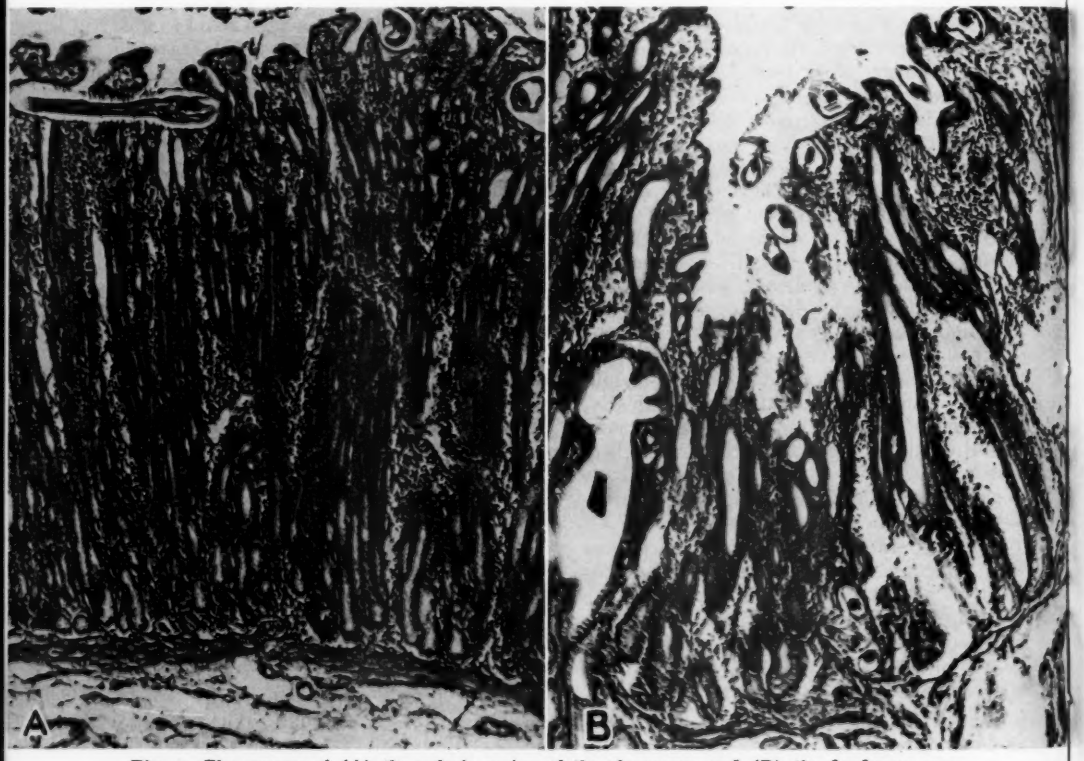

Fig. 3—The mucosa of (A) the pyloric region of the abomasum, and (B) the duodenum near the pyloric valve of lamb 1, showing typical histopathologic changes observed at necropsy in acutely affected lambs. Notice the worms in situ, edema, cellular infiltration, and the destruction of the superficial mucosal epithelium.

In conclusion, the available facts indicate that *T. axei* has a high potential for pathogenesis in ruminants, and the disease entity that is caused by this parasite is one of considerable complexity.

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1) Five lambs, given 50,000 to 300,000 *Trichostrongylus axei* each as single doses, died, or were *in extremis*, 30 to 42 days later. The lamb given 500,000 larvae survived and was less severely affected than the others, probably as a result of developing only a low level patent infection.

2) The clinical effects shown by the 5 fatally infected lambs were anorexia, acute diarrhea, emaciation, physical weakness, rapid and marked loss of weight, dehydration, and a terminal relative polycythemia. In the surviving lamb most of these effects were less pronounced, diarrhea did not occur, and a slight terminal anemia developed.

3) The number of worm eggs per gram of feces attained an average maximum of more than 7,000 in the 5 fatally infected lambs, whereas a maximum of only 84 was recorded from the surviving lamb.

4) The carcasses of the 5 fatally infected lambs were emaciated and dehydrated and the blood was dark red and viscous. Acute abomasitis and duodenitis, with noticeable enlargement of the abomasum, were consistent pathologic changes observed in these lambs. In the surviving lamb, these changes were much less marked.

5) The numbers of worms recovered from these lambs varied from 5,720 to 45,862, and were inversely related to the quantities of larvae given.

6) Histologic study of abomasal and duodenal tissue of the infected lambs revealed erosion of the superficial mucosa, edema, hyperemia, and lymphocytic infiltration. A few adult worms were observed in the glands and mucosal tissue.

7) The present status of *T. axei* as a pathogen of ruminants is discussed in relation to recent important contributions of other workers.

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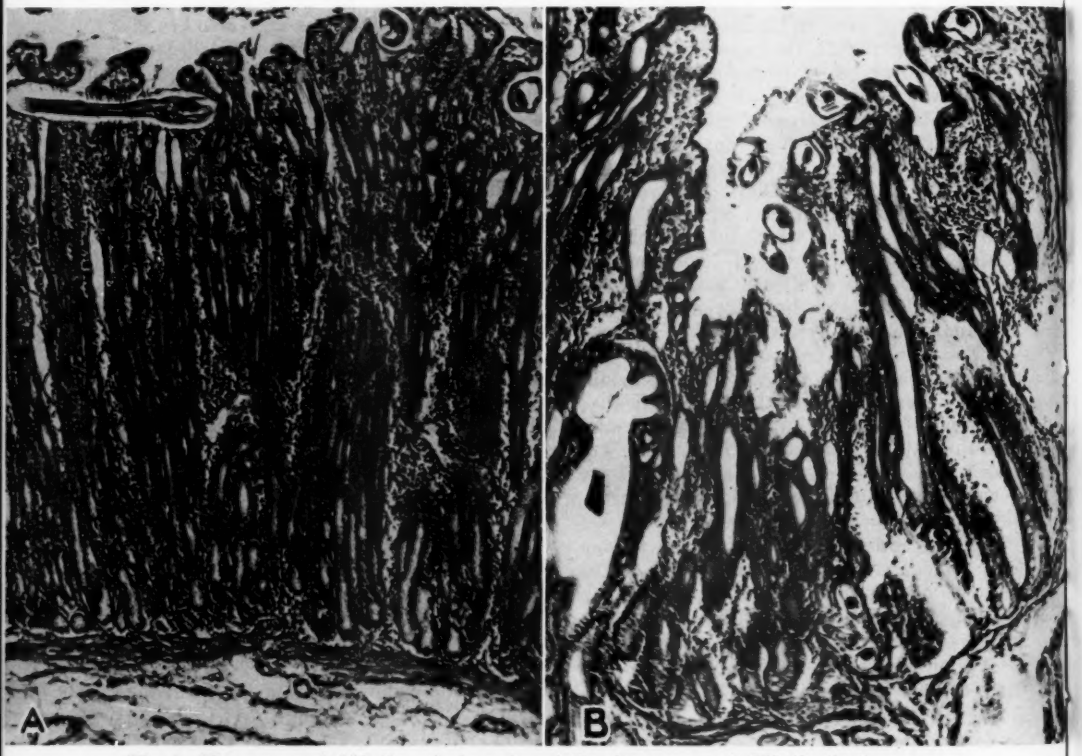

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Experimental Infections of Swine with *Trichuris suis*

K. G. POWERS, M.S.; A. C. TODD, Ph.D.; S. H. McNUTT, D.V.M.

Madison, Wisconsin

TRICHURIS SUIS, Schrank, 1788, is a common parasite of young swine in Wisconsin,[11] but trichuriasis has not been regarded as a serious parasitic problem within the swine industry, nor has the parasite been considered a particularly pathogenic organism.[9] Other workers consider certain whipworm species capable of causing severe disease and even death.[6, 8, 10]

The whipworm frequently is recovered in mixed infections with *Ascaris lumbricoides*. Evidently the signs of trichuriasis may be masked by or confused with those of ascariasis.

Two trials have been completed to determine the course and pathogenicity of *T. suis* in pigs under experimental conditions. The results of these studies stimulate an interest in a re-evaluation of the clinical importance of this parasite.

MATERIALS AND METHODS

The 6 pigs used in these trials were purebred Spotted Poland Chinas, farrowed in rigid isolation to preclude possible parasitic infections. They remained with the dam until weaned at 8 weeks of age. Two then were separated as controls and placed in another unit; 4 were utilized as test animals. All 6 were fed a grain mixture in self-feeders.

Whipworm ova used in the exposures were obtained from a "seed pig." Manure from the seed pig was cultured at room temperature in a moist medium consisting of sterile manure, powdered bone charcoal, and peat moss. Fully embryonated ova developed in 22 days. Infective ova were suspended in water and administered by means of a stomach tube.

Experiment 1.—Four of the pigs, at the age of 78 days, were exposed to infective *T. suis* ova over a 19- to 22-day period (table 1). This method of multiple exposure was employed to simulate natural conditions. Total exposure of each of the 4 was estimated at 39,700, 34,500, 34,500, and and 50,000 infective ova respectively.

All animals were weighed at weekly intervals after the initial exposure.

At necropsy, tissues were preserved and later processed for histopathologic studies. Tissues were not taken from 2 pigs; they died during the night and their tissues were presumed unfit for sectioning. Worms recovered from each animal were counted individually, and their distribution in the cecum and colon was plotted.

Experiment 2.—An attempt was later made to initiate similar infections in the 2 control animals when 160 days old and near market weight. Each was exposed to 34,500 infective ova over a period of 19 days (table 1). Depending upon the availability of fecal samples, counts of ova were made from the 25th day after initial exposure until the animals were killed. The animals were weighed weekly and their body temperatures recorded twice daily. Infected and control hogs were necropsied and tissues were removed for sectioning.

RESULTS AND OBSERVATIONS

Experiment 1.—The appetites of all 6 pigs apeared to be "normal" until experimental day 26. Anorexia then developed gradually in 3 animals but the fourth showed no signs of infection. Two of the 3 affected pigs died on experimental days 43 and 44, respectively; the third was killed when moribund on day 53.

The control pigs remained apparently healthy.

Although the pens were cleaned twice daily, the 3 affected pigs developed a generally soiled appearance resulting from bloody diarrhea. Approximately on experimental day 33, these animals appeared anemic, with pallid oral and conjunctival mucous membranes. They showed emaciation, weakness, and unsteady gait. On day 40, prolapse of the rectum was observed in 1 animal, evidently a result of trichuriasis.

Fecal Consistency.—There was considerable variation in consistency and amount of feces passed by the 3 individual animals throughout the trial period. Diarrhea was first observed in all 3 pigs on experimental day 19. Constipation was noticed in 1 pig on day 22 and in the second pig on day 25. On day 25, the first animal passed soft semiliquid stools; the third pig passed soft

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blood-flecked feces. All 3 infected animals had watery, bloody feces which changed to a bloody mucoid discharge a few days prior to death.

Effect on Growth.—Definite differences in weight gains between the controls and the infected animals were first observed during the third week (fig. 1). The affected animals had been gaining, but about the sixth week they began to lose weight. A marked weight loss preceded their death, when they weighed, respectively, 70, 81, and 73 lb. less than the average of the 2 controls.

Gross Pathologic Changes.—Evidence of peritonitis was observed about the cecum and colon in 1 animal. A large number of *T. suis* was found in the lumen and attached to the mucosa of the cecum and colon; worms were found throughout the entire length of the colon. In both organs there was apparent necrosis, edema, and hemorrhage of the mucosa. The mesenteric lymph nodes were not enlarged. Congestion was observed in the fundic region of the stomach and near the pyloric sphincter. The liver was mottled. Bronchial and mediastinal lymph nodes were hemorrhagic, congested, and swollen. Grossly, the lungs, heart, spleen, kidneys, and the voluntary muscles appeared normal.

Pathologic changes in the other 2 affected animals were similar to those just described, including those of the cecum and colon, except that peritonitis was not observed. Areas of atelectasis and lobular emphysema were seen in 1 animal. The small intestine, heart, spleen, kidneys, and liver of both were apparently normal.

Histopathologic Changes.—Examination of sections of the colon revealed worms and worm ova on the surface and, not infrequently, deep within the mucosa. Penetration by *T. suis* via the crypts of Lieberkühn as far as the muscularis mucosae has been reported.[2] Catarrhal inflammation with its associated surface exudate was seen, perhaps a result of irritation caused by the worms. Cellular infiltration with lymphocytes and with what appeared to be plasma cells was observed. Eosinophils were prominent. The epithelial cells in the area immediately surrounding the worms were observed to be misshapen and shrunken to some extent. Some of the glandular cells contained excessive amounts of mucus.

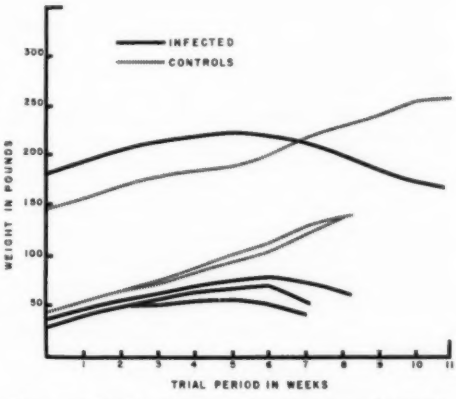

Fig. 1—Weight gains of pigs with pure Trichuris suis infections.

Generally, the presence of the worms seemed to incite an inflammatory reaction, *i.e.*, dilation of the blood vessels, lymphocytic infiltration, edema, and excessive mucus formation.

TABLE 1—Experimental Exposure of Swine to Trichuris suis Ova

Pig No.	Age when exposed to initial infection (days)	Period of exposure (days)	Estimated total of infective ova administered	Prepatent period (days)	Time from initial exposure until necropsy (days)	Number of worms recovered	Percentage of worms to number of infective ova administered (%)
1*	78	....	.....	....	....	.....	....
2*	78	....	.....	....	....	.....	....
3	78	20	39,700	**	45	3,609	9
4	78	19	34,500	**	44	11,863	34
5	78	19	34,500	44	53	10,866	32
6	78	22	50,000	No "take"	74	.....	....
1	160	19	34,500	....	77	.....	....
2	160	19	34,500	41	77	13,211	38

* Controls, no infective ova given; ** died before nematodes matured.

Histopathologic changes extended to the deeper glands within close proximity to the muscularis mucosae. These glands frequently were cystic and distended, apparently with epithelial cells in various stages of disintegration. Hemorrhagic areas were seen below and around the muscularis mucosae, but the origin of the blood could not be determined. Eosinophils were scattered throughout this area.

Intestinal ulcers were present in areas containing the submuscularis lymphoid tissue. Although these were small, they extended into the lymphoid tissue.

The colic lymph nodes were congested and hemorrhagic with areas of greater or less involvement. The subcapsular and peritrabecular sinuses were distended with erythrocytes and eosinophils. Numerous eosinophils were diffused throughout, and monocytes were abundant in the deeper portions of the lymph node.

A few hemorrhagic areas were seen in the kidneys. Reticular hyperplasia was noticed in the spleen. The liver and lungs were without apparent change.

Counts of Ova and Worm Recovery.—Ova were not found in the feces of the 2 animals which died on experimental days 43 and 44. The feces of the third pig contained 1,265 ova per 5-Gm. sample on day 45. The number of ova passed by this animal varied from 1,265 to 169,000 per 5-Gm. fecal sample.

No ova were passed in the feces of the 2 control animals during the experiment.

The number of infective ova administered to each animal and the number of worms recovered at necropsy are recorded (table 1). Two animals were exposed to the same number of infective ova, and approximately the same number of worms were recovered from each. The percentage of worms recovered to number of infective ova given was 34 and 32 per cent, respectively. The third animal, although given 5,200 more infective ova than the other 2, yielded only 3,609 worms, a recovery of 9 per cent.

The decline in percentage of helminth development associated with an increase in numbers of ova in experimental exposures has been reported to occur with *Ascaridia lineata* infections in chickens and with *Trichostrongylus axei* infections in rabbits.[1,7]

Experiment 2.—The same ration was given to these animals as when they were controls in experiment 1.

Following exposure (34,500 ova in 19 days), 1 animal developed no apparent signs of trichuriasis and was utilized as the "control." Throughout the study, its temperature range remained between 100.9 and 102.7 F. It failed to acquire a true final infection and no worms were recovered at necropsy.

The second animal developed an increased temperature, on day 24, continued to a peak of 104.1 F. on day 58 then decreased but remained slightly above that of the control until day 71 when it dropped to 99.8 F. Anemia was first observed on day 40. The animal was more listless by

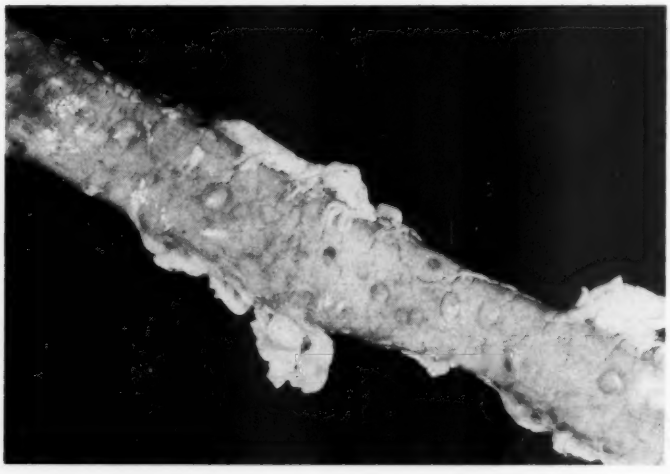

Fig. 2—Nodules on an unopened portion of the colon (peritoneal surface).

day 71 and was moribund on day 75. Partial anorexia was first observed on day 32 and persisted to day 62. Complete anorexia then developed and persisted until the animal was killed in a moribund condition on day 77.

The consistency of the feces of this animal was similar to that of the pigs in experiment 1. It was characterized by diarrhea which progressed from soft, blood-flecked feces on experimental day 29 to bloody-watery stools on day 32 and terminated in a bloody-mucoid discharge 12 days prior to death on day 77.

Numerous specimens of *T. suis* were passed by this animal during experimental days 53 to 55, 61, 66, 67, and 69 to 73. Including those recovered at necropsy, the total yield was 13,211 worms, representing 38 per cent of the ova given.

The number of ova in the feces ranged from 4 to 47,320 per 5-Gm. sample. A pattern of ova production characterized by a sharp rise followed by a sharp drop was observed to be typical of this parasite. The peaks in ova production occurred on experimental days 43, 55, 59, 67, and 74. Based on a prepatent period of 41 days, these peaks seemed to coincide with maturity of the worms following each exposure.

Experimental trichuriasis produced deleterious effects on the growth rate of the affected animal (fig. 1). The most rapid weight loss was recorded during the seventh week. When killed, the weight difference between the control and the affected animal was 87 lb.

Gross Pathologic Changes.—The pathologic changes resembled those seen in the pigs in experiment 1, with some additional findings. Two distinct types of nodules were seen within the walls of the cecum and colon (fig. 2, 3). One was soft and contained pus; the anterior portion of a worm usually was embedded deep within the process (fig. 4). The second was a firm, encapsulated, rounded mass beneath the mucosal surface. Numerous white foci were seen on the parietal surface of the liver. The gallbladder was distended with thick, dark-green bile. The bronchial lymph nodes were congested and enlarged.

Examination of the viscera in the "control" animal revealed the presence of numerous ulcer-like lesions in the cecum and colon, apparently having resulted from the larvae burrowing into the mucosa, although no worms were recovered. No lesions were observed in the other organs.

Histopathologic Changes.—These changes were largely similar to those in the younger pigs reported in experiment 1. There were the same cystic intestinal glands and the same catarrhal inflammation associated with the hemorrhagic areas, both of which were more pronounced. Intestinal ulcers and nodules were associated with parasitic invasion of the lymphoid tissue (Peyer's patches), with resulting lymphoid hyperplasia. Liquefaction did not occur in the firm or "hard core" parasitic nodules,

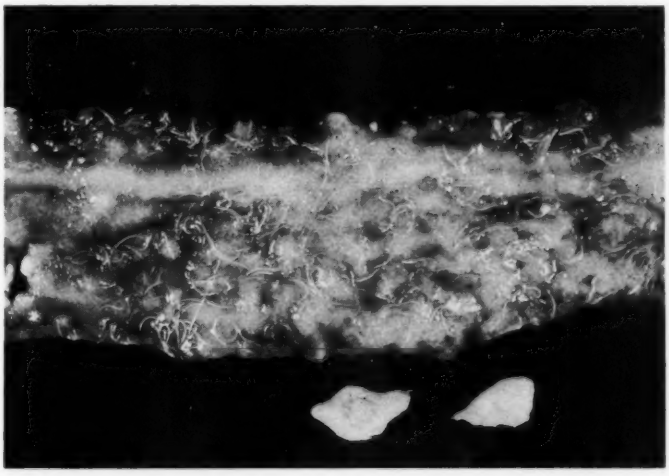

Fig. 3—Worms and nodules on the mucosal surface of same section of colon shown in figure 2. Isolated pus-containing nodules are shown below the intestine.

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which were granuloma-like and contained the anterior portions of the worm body, ova, phagocytes, and necrotic detritus (fig. 5).

The examination of a mesenteric lymph node from this affected pig showed it to be more hemorrhagic, with sinuses more distended, than in like lymph nodes of the younger pigs. No eosinophils were observed.

Small congested regions were observed in the duodenum close to the pyloric sphincter.

The course and pathogenesis of the infection in the 160-day pig closely resembled that in the younger 78-day-old animals. The prepatent period in the older animal was 41 days, compared with 45 days in a younger pig. The percentage of worms recovered at necropsy in the 78-day pigs was 9, 34, and 32 per cent respectively, compared with 38 per cent in the 160-day pig. Bloody diarrhea appeared in animals of both age groups at approximately the same number of days after initial exposure, *i.e.*, day 29 in the older animal and days 25 to 27 in the younger pigs. Inappetence developed on day 32 in the 160-day pig, while it occurred on day 26 in the younger pigs. At death, a weight difference of 87 lb. was found between the control and the older infected animal, relatively less but similar to the pronounced weight loss observed in the younger pigs.

DISCUSSION

From this study, it can be tentatively concluded that *Trichuris suis* is not a harmless commensal but rather a pathogen capable of causing severe damage. The extent of injury to the host produced by *T. suis* in natural infections evidently has been masked in part by ascariasis. Moreover, these experimental infections established that the parasite has a rather short life span, seldom exceeding four to five months. These conclusions are supported by surveys on young and market-age animals. In such a survey of Dane County, Wisconsin, it was found that 75.5 per cent of 8- to 24-week-old pigs were infected, contrasted to 13.9 per cent in market swine.[5,11] It is evident that the host acquires an appreciable degree of resistance to the infection. Frequent reexposure obviously is possible.

These experimental infections demonstrated the prepatent period of *T. suis* to vary from 41 to 45 days, somewhat less than the 45- to 47-day prepatent period reported.[10] A more prolonged prepatent period extending to 90 days also has been reported.[9]

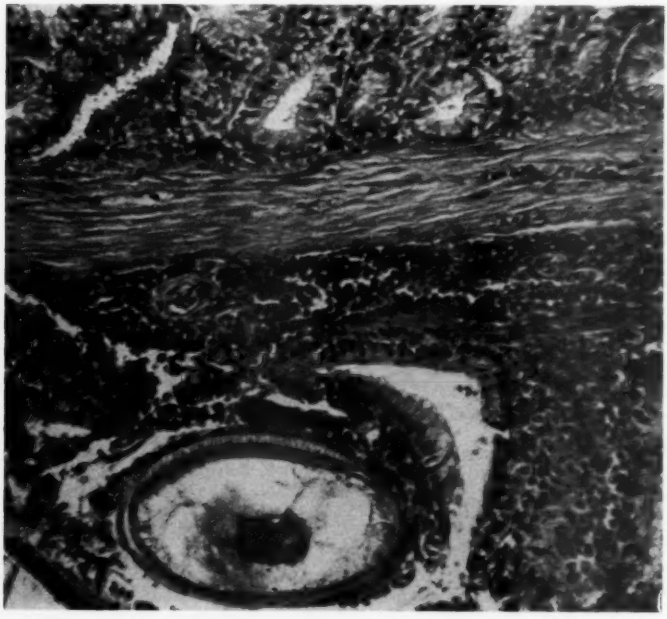

Fig. 4—Cross section through a soft, purulent nodule showing its relation to the muscularis mucosae. Notice lymphocytic infiltration around the sectioned anterior portion of the worm. x 250.

The greatest number of ova passed by any one animal artificially infected was 169,000 per 5-Gm. fecal sample. The pattern of production in "heavy" infections was characterized by a rise in production reaching a peak about two weeks after the first appearance of ova. This peak subsequently was followed by a rapid decrease. Because of such a rapid and early decrease in egg production, care must be exercised in the testing of trichuricides. Satisfactory results might incorrectly be attributed to a test drug if efficacy were based on number of ova passed when the decrease in production was no more than a physiologic phase in the parasite's life cycle.

The percentage of worm recovery was not directly related to the observed pathologic changes. One of the younger animals, with a low percentage worm recovery, exhibited changes similar to those in animals from which a greater number of worms was recovered. Individual host resistance may have been a contributing factor.

In the 78-day-old pigs, a "recovery" from a period of weight loss was seen, followed by other decreases in weight gain. Recovery and maintenance of weight may correspond with the emergence of the larvae from the mucosa, and final maturity of the worms.

Anemia apparently resulted from the hemorrhagic enteritis and the blood-sucking habits of the worms. Vibrio spp. also has been reported to cause a bloody enteritis in swine.[3]

Nodules were believed to result from penetration by the adult worm. The host response was an inflammatory reaction which resulted in encapsulation of the process, including a part of the parasite. Abscess formation in the cecum and colon resulting from secondary infection following Trichuris invasion has been reported.[4]

Trichuris suis was found in the colon as well as the cecum. The worms had a predilection for the upper third of the colon, but the entire organ was a satisfactory habitat for the worms since they were recovered as far posteriorly as the terminus of the rectum.

It was considered possible that the dysentery observed in the infected animals might have been produced by another pathogen. Therefore, when the animals were passing bloody feces, their stools were examined for Salmonella; none was recovered. Vibrio-like organisms were found in smears taken from the colon at necropsy of an infected

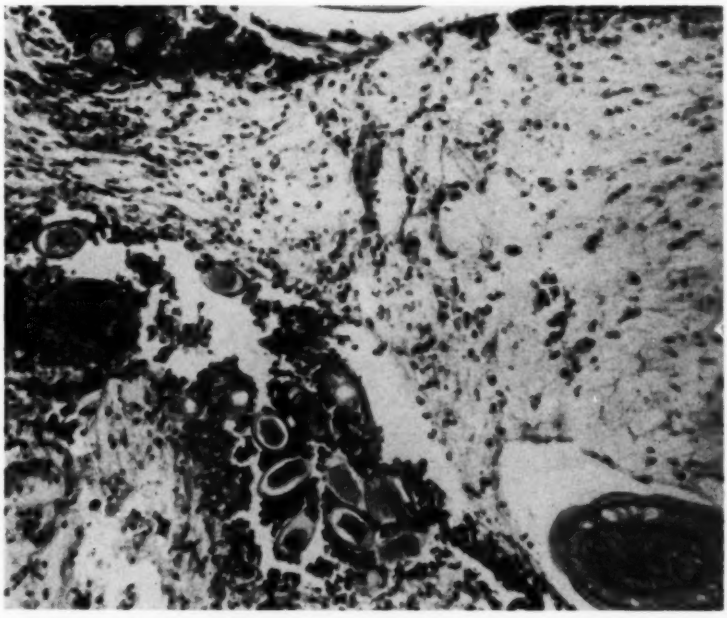

Fig. 5—Portions of the worm body and ova contained within a hard-core nodule. Notice lymphocytic infiltration and areas of necrosis. x 250.

pig. In a transmission experiment, on a pig without previous Vibrio or nematode infections, bloody feces from the experimental pigs were fed repeatedly to a pig in isolation with negative results for a month after exposure. Neither a Salmonella nor a pathogenic Vibrio was demonstrated in these experiments.

SUMMARY AND CONCLUSIONS

1) Studies have been made on the prepatent period, course of production of ova, and longevity of *Trichuris suis* in experimental infections. The prepatent period was found to be 41 to 45 days and the life span 4 to 5 months. Production of ova was characterized by a sharp rise followed by a rapid fall.

2) Clinical manifestations of experimental trichuriasis included anemia, anorexia, dysentery, and pronounced weight losses, regardless of age.

3) *Trichuris suis*, like Oesophagostomum spp., can cause "nodular disease" in swine.

4) *Trichuris suis* penetrates the intestinal mucosa with considerable destruction and the formation of nodules in the intestinal wall. Clearly, *T. suis* also can act as a provoking factor for secondary invasion with other disease-producing agents.

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Isolation and Cultivation of Swine Pox Virus in Primary Cell Cultures of Swine Origin

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THIS REPORT describes the application of tissue culture techniques for the isolation and study of the swine pox virus.

Two viruses have been isolated from pock lesions of swine.[4,6,7,8] One is similar to or identical with the vaccinia virus, and the term, "vaccinia infection of swine," has been used when referring to swine so infected. The second virus is immunologically distinct[4,6] from the other known pox viruses, is infectious only for swine,[6] and is the agent causing the disease commonly referred to as swine pox.[6,7,8] Both viruses usually produce benign diseases in swine, manifested mainly by the appearance of pock lesions on the abdomen and inguinal regions. The louse, *Haematopinus suis*, plays an important role as a vector of the swine pox virus.[4,6]

Although there are several reports on the use of tissue culture techniques for the study of pox viruses (vaccinia,[2] variola,[10] and the sheep pox virus[5]), none could be found for the swine pox virus.

MATERIALS AND METHODS

Cell Cultures.—Primary monolayer cultures were prepared by the trypsinization of the specified tissue with the subsequent growth of the dispersed cells in a culture medium consisting of: 0.5 per cent lactalbumin enzymatic hydrolysate; 5 per cent bovine serum; 94.5 per cent Hanks' balanced salt solution (BSS); and 100 μg. of streptomycin, 200 units of penicillin, and 50 units of nystatin (mycostatin*) per milliliter. The porcine kidneys usually originated from 5- to 7-month-old swine.

Plaque Technique.—Monolayer cultures of porcine kidney cells in 4-oz. prescription bottles were prepared and used as previously described.[9]

Strains of Infected Tissue Culture Cells.—Porcine kidney cells were grown on cover slips, 10 x 50 mm., in Leighton-type tubes. These cells were then infected with the swine pox virus and after a cytopathogenic effect (CPE) was noticed, the cover slips were removed from the tubes and the cells were fixed in Zenker's solution, and stained with Harris' hematoxylin and eosin.

Neutralization Tests.—The serums used for conducting the neutralization tests were obtained from animals inoculated as follows: Two pigs, 12 weeks old, were infected by scarifying the skin and applying swine pox virus of the fifth tissue culture passage. Both pigs gave typical reactions and, 16 days postinfection, each was reinoculated with the same virus, 1 ml. intravenously and 1 ml. intramuscularly. Blood samples were obtained from these pigs 23 days after the original inoculation and their serums pooled. Each of 2 rabbits was inoculated with the same pool of virus, 1 ml. intravenously and 1 ml. intramuscularly, with the former injection repeated five and nine days later. Blood samples were obtained from them 16 days after the original injection and the serums pooled.

The swine and rabbit serum samples were each diluted 1:8, 1:16, and 1:32 with BSS and mixed in equal volumes with the swine pox virus of the sixth tissue passage, diluted so as to give 16 50-per cent tissue culture dose ($t.c.d._{50}$) per 0.1 ml. Following incubation at room temperature for one hour, 0.4 ml. of each of the virus-serum mixtures were inoculated into each of three 4-oz. prescription bottles containing monolayer cultures of porcine kidney cells. These bottles were microscopically examined daily for eight days for evidence of a CPE.

EXPERIMENTAL PROCEDURE AND RESULTS

Virus Isolation.—Approximately 20 per cent of the animals in a herd of 300 young pigs, 2 to 4 months of age, showed evidence of pox infection. Most of the pigs were also infested with lice *(Haematopinus suis)*. About 20 per cent of the visibly infected pigs were retarded in growth. The majority of the pock lesions were located on the abdomen and inguinal region.

Encrusted material from approximately 15 pock lesions and 100 lice, obtained from several of the infected pigs, were thoroughly ground together in a mortar so as to make a 5 per cent suspension in BSS. To each milliliter of this suspension was added 1,000

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* Mycostatin is produced by E. R. Squibb and Sons, New York, N.Y.

μg. of dihydrostreptomycin, 2,000 units of penicillin, and 500 units of nystatin. After centrifugation at 4,000 r.p.m. for 30 minutes, 0.2 ml. of the supernatant fluid was inoculated into each of four 4-oz. prescription bottles containing monolayer cultures of porcine kidney cells. After seven days no CPE was evident, whereupon the cells were scraped from the walls of the bottles and inoculated into other bottles containing porcine kidney cells. The second and third tissue culture passages using cells collected after 11 and 14 days, respectively, were made in a similar fashion but with no evidence of a CPE. On the fourth blind passage a CPE was noticed on the sixth day with an almost complete destruction of the cells occurring by the ninth day. Fluid was harvested and upon subsequent passages a (CPE) was usually first noticed after three to five days.

Cytopathogenic Effect.—The CPE of this swine pox virus on a monolayer of porcine kidney cells is illustrated (fig. 1) and compared with an uninfected culture of cells (fig. 2). Usually some cells in a culture resisted the destructive effect of the virus.

A particularly outstanding feature of the infection of these cells was the development of sizeable and distinct intranuclear "vacuoles" which were demonstrable upon staining with hematoxylin and eosin (fig. 1, 3). These intranuclear vacuoles, which took no stain and which appeared to be merely empty spaces, were the first visible evidence of infection. Usually only one, but occasionally two, were seen in a nucleus.

Cytoplasmic inclusions were present but were variable in size, indistinct in outline, granular in appearance, and not always evident in the infected cells. They were usually eosinophilic, although occasionally neutrophilic and, in the larger ones, denser basophilic spots were evident.

Plaque Formation.—Plaques were produced on monolayers of porcine kidney cells. They first became evident about five days after virus inoculation and never increased much in size, remaining less than 1.5 mm. in diameter. The titer of the virus as determined by the number of plaques produced paralleled that obtained by the detection of CPE in tissue culture tubes.

Infectivity for Animals and Tissue Culture Cells.—Three pigs, 12 weeks old, were inoculated by the scarification technique in the inguinal and abdominal areas, using the virus from the sixth tissue culture passage. Two days later, the inguinal lymph nodes became palpable and the temperatures of the pigs were elevated about 0.5 F. above those of the control animals in the same

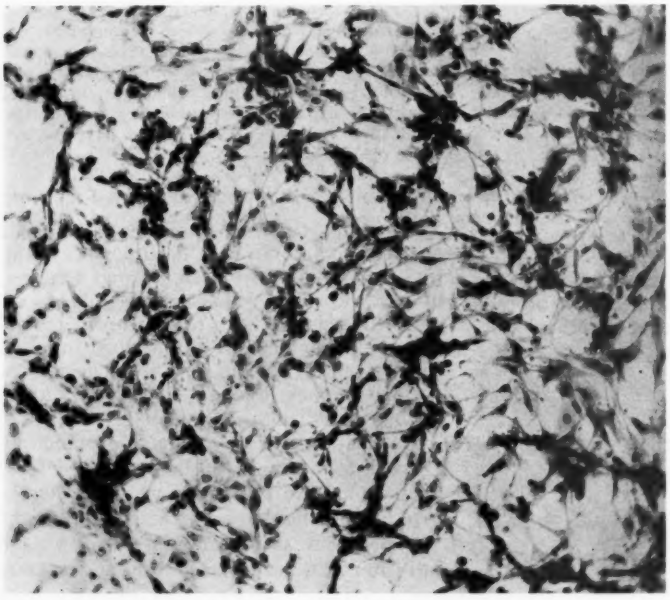

Fig. 1—Porcine kidney tissue culture, five days after inoculation with swine pox virus of the fifth tissue culture passage. H & E stain; x 150.

room. On the third day, the scarified areas were slightly swollen and erythematous. The lesions progressed to papules and on the sixth day became vesicular, changing to pustules about two days later. The pustules gradually became dryer and changed from brown to black (fig. 4). During the vesicular and early pustular stages the pigs were slightly depressed, had temperatures of 102.0 to 103.5 F., and had only moderate appetites.

Twelve days postinfection, pustules were collected from these pigs and prepared as described for inoculation into bottles of porcine kidney cells. A CPE was evident in these cells on the fifth day with most of the cells showing infection two days later.

One pig, approximately 12 weeks old, was inoculated intravenously with 1 ml. of swine pox virus of the sixth tissue culture passage. On the sixth day postinoculation, lesions on the skin became evident. Approximately 150 pox lesions occurred, scattered over the body, with the majority of them in the abdominal and inguinal regions. The only observable systemic reaction was a loss of appetite on the sixth day.

The virus from the fifth tissue culture passage was inoculated onto the "dropped" chorioallantoic membranes of 12-day chicken embryos. These membranes were harvested four days later and they appeared normal, but were ground and reinoculated similarly onto the chorioallantoic membranes and into tubes of porcine kidney cells. Again, no lesions developed on the membranes and no CPE was observed in the tissue culture cells, suggesting that the virus did not survive its sojourn on the chorioallantoic membrane.

The virus from the fifth tissue culture passage was deposited onto the scarified skin of 2 rabbits and 2 guinea pigs, and injected both intravenously and intramuscularly into 2 other rabbits. No local or systemic reactions were observed in any of the animals.

Tissue culture cells from a variety of sources were inoculated with the virus from the fifth tissue culture passage. A CPE was readily apparent on cells derived from porcine kidneys, embryo lungs, embryo brain, and testes. The $t.c.d._{50}$ per 0.1 ml. of the virus grown on cells from porcine kidneys was usually $10^{4.0}$. The cells grown from porcine kidneys were primarily epithelial-like, while those from the testes were fibroblastic. The virus produced a CPE equally well on either type of cell. Twelve days after the inoculation of bovine kidney cells, no CPE was evident. The cells were then scraped from the glass wall and trans-

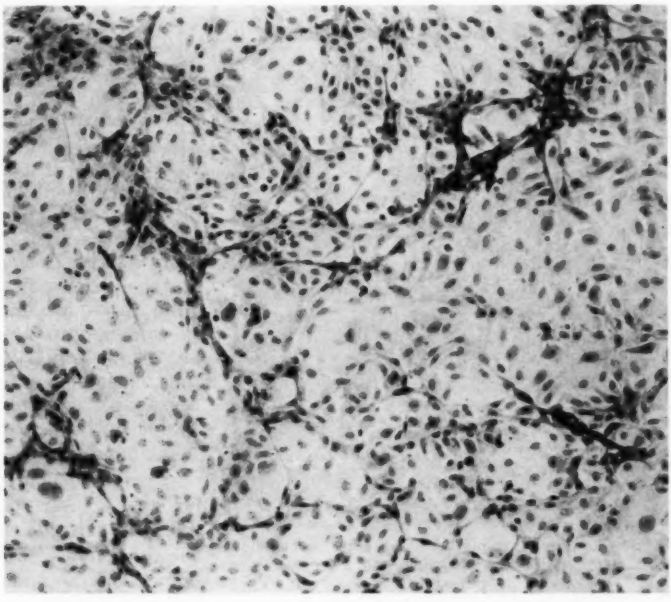

Fig. 2—Porcine kidney tissue culture, uninfected. H & E stain; x 150.

Fig. 3—Porcine kidney tissue culture, four days after inoculation with swine pox virus of the fifth tissue culture passage. H & E stain; x 1485.

ferred to a culture of porcine kidney cells. A CPE was evident after six days, indicating that the swine pox virus had survived in the presence of bovine kidney cells. No CPE was evident in the culture of rabbit kidney cells.

Neutralization Tests.—The only evidence of a neutralizing effect by the porcine and rabbit serum samples on the swine pox virus was a 24-hour delay in the CPE at serum dilutions of 1:8 and 1:16. Seven days after inoculating the tissue cultures with the various serum-virus mixtures, the CPE was similar to that observed in the virus control bottles. Therefore, our techniques did not demonstrate any appreciable neutralizing effect by the serums on the swine pox virus.

Three pigs that had been artificially infected 30 days previously with swine pox virus of the fifth tissue culture passage were again exposed to the swine pox virus by the skin scarification technique. None of these pigs showed lesions of swine pox.

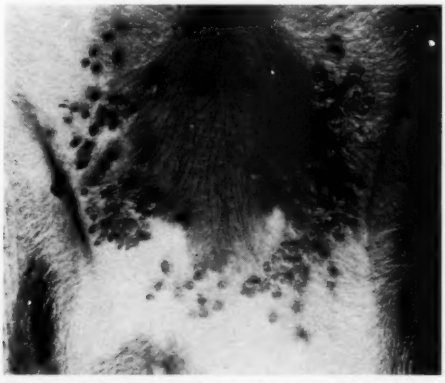

Fig. 4—Lesions on a pig ten days after inoculation with swine pox virus of the sixth tissue culture passage.

DISCUSSION

A clinical diagnosis of swine pox was made in a herd of swine in Ohio. From the pustules and lice collected from infected pigs, a virus was isolated by the use of tissue culture methods employing porcine kidney cells. However, a CPE was first noticed only after the fourth blind passage but was consistently present on subsequent passages. The virus from the sixth tissue culture passage produced typical lesions of swine pox when inoculated into 12-week-old pigs. Pustules from these artificially infected pigs produced a CPE upon primary inoculation onto porcine kidney cells, which was in contrast to what had been observed in regard to the isolation of the virus from the naturally infected pigs. The virus had apparently retained its newly acquired ability to produce a CPE on porcine kidney cells even after a passage in a susceptible pig.

From the tests so far conducted, this virus has been able to infect only swine or cells from swine. All of these characteristics of the virus would seem to justify its identification as that of the etiological agent of true swine pox. It definitely is not vaccinia virus, which has also been reported as causing pox lesions in swine, since no lesions were produced in rabbits, guinea pigs, or embryonating chicken eggs nor was there any CPE on monolayer cultures of rabbit or bovine kidney cells.

A characteristic feature of the infection of monolayer cultures of porcine kidney cells with this virus was the formation of distinct intranuclear "vacuoles." Whether these vacuoles should be called inclusion bodies may be open to question. Plowright

and Ferris [5] observed similar intranuclear vacuoles in tissue culture cells infected with sheep pox, but hesitated to call them inclusion bodies since no distinct formed body was demonstrable. Blakemore and Abdussalam [1] reported the presence of round or ovoid intranuclear inclusions in epidermal cells in the early stages of swine pox. Their photographs of these intranuclear inclusions bore a striking resemblance to the intranuclear vacuoles which we have observed in infected tissue culture cells.

Confirming a previous report of Shope,[6] serums from hyperimmunized pigs and rabbits failed to neutralize, *in vitro*, the swine pox virus. However, as has also been reported by Shope,[6] pigs that had recovered from a swine pox infection were resistant to a reinfection with the same virus. Apparently the swine pox virus can be effectively neutralized *in vivo* but not *in vitro*.

SUMMARY

1) A virus, identified as the swine pox virus, was isolated from pock lesions of swine and associated lice by the use of monolayer cultures of porcine kidney cells.

2) On the fourth blind passage in porcine kidney cells, the virus first produced a cytopathogenic effect characterized by the formation of intranuclear "vacuoles" with the eventual destruction of most of the cells. Cytoplasmic inclusion bodies were also observed. The virus also produced a cytopathogenic effect on porcine cells derived from testes, embryo lung, and embryo brain.

3) The virus produced no lesions on the chorioallantoic membranes of embryonating chicken eggs or on the scarified skin of rabbits or guinea pigs. There was no cytopathogenic effect on monolayer cultures of bovine or rabbit kidney cells.

4) Typical pock lesions were produced in swine by inoculating virus of the sixth tissue culture passage intravenously or by skin scarification.

5) Very small plaques were produced by the virus on monolayer cultures of porcine kidney cells.

6) Serums from hyperimmunized pigs and rabbits failed to neutralize, *in vitro*, the swine pox virus. However, recovered pigs were resistant to reinfection with the swine pox virus.

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Serotypes of Avian Pleuropneumonia-Like Organisms

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THE ROLE of pleuropneumonia-like organisms (PPLO) in avian disease has been the subject of considerable discussion since 1952, when Van Roekel and Olesiuk [20] and Markham and Wong [17] suggested their relationship to the chronic respiratory disease (CRD) of chickens described by Delaplane and Stuart [5] and infectious sinusitis (IS) of turkeys.[6] Further speculation was prompted by the similarity of these organisms to the coccobacilliform bodies described nearly 20 years earlier by Nelson [18] as the causative agent of a coryza-like disease of chickens. Subsequently, other workers concurred in the opinion that PPLO were at least essential, if not the exclusive, etiologic agents of CRD and IS.

Efforts were early directed toward serologic and culture diagnosis of CRD and IS. Jungherr *et al.*[12] reported on the application of the PPLO hemagglutination-inhibition (HI) test previously described by Van Herrick and Eaton.[19] Adler [1] developed a rapid slide agglutination test for diagnosis of the PPLO of CRD and IS. The usefulness of serologic tests was further stimulated by the studies of Fahey,[7] White *et al.*,[21] and Gianforte *et al.*,[8] which indicated that their strains of PPLO from CRD and IS were antigenically and morphologically homogeneous.

Following the successful cultivation of avian PPLO on artificial culture mediums,[17,20] attempts were made to simplify the mediums and procedures involved.[2,9,10,15] Although PPLO were readily isolated from avian tissue, many of the isolates possessed little, if any, pathogenicity and possibly also differed serologically,[3,13,16] suggesting that both pathogenic and nonpathogenic PPLO existed in avian tissues. It was further observed that nonpathogenic PPLO could be egg-transmitted.[13]

Since it was not known what, if any, relationship existed between the pathogenic and so-called nonpathogenic PPLO, it was likewise not known what effect the presence of nonpathogenic PPLO in the bird might have on the serologic tests for the detection of CRD and IS. It was, therefore, decided to undertake a study on the antigenic relationship of avian PPLO and to correlate this with other biologic characteristics of the organisms.

MATERIALS AND METHODS

Avian PPLO isolates were assembled from various geographical sources for this study (table 1). Egg-yolk cultures were maintained by occasional egg passage and were stored as frozen yolk cultures at −60 C. for periods up to four years. Isolates obtained or received in artificial culture mediums were maintained by periodic transfer and interim storage in artificial culture mediums at −60 C. During the course of this study, Adler *et al.*[4] reported on strain differences and the characterization of five PPLO types from diseased and normal birds. Six strains representing his five types were subsequently included in the studies reported here (table 1).

Throughout this study, all isolates were cultivated and maintained in a liquid medium consisting of Difco PPLO broth (without crystal violet) to which was added 1 per cent yeast hydrolysate (pH readjusted to 7.6), 1 per cent Difco PPLO serum fraction, thallium acetate in final concentration of 1:2,000, and 1,000 units of penicillin per milliliter, respectively.

Antigen Production.—Seed cultures for antigen production were prepared by inoculating approximately 5 ml. of stock culture (5–7 days' incubation) into flasks containing 100 ml. of broth. After five days' incubation, the contents of one flask were seeded into 1 liter of broth contained in 5-liter Pyrex diphtheria toxin bottles. A production lot consisted of 6 bottles (6 liters) of medium. The bottles were gently rocked on a platform-type mechanical shaker during the incubation period of five days at 37 C. The cells were harvested by centrifugation in a refrigerated, steam-driven super centrifuge,* using the "virus bowl," at 50,000 r.p.m. The cells were removed from the bowl and washed three times in sterile buffered

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* Sharples Super Centrifuge, manufactured by the Sharples Corp., Philadelphia, Pa.

saline solution (pH 7.2). They were packed by centrifugation at 6,000 r.p.m. for one hour in a refrigerated centrifuge† at 1 C. and stored at 4 C.

Production of Hyperimmune Rabbit Serums.—All antigens were adjusted to a concentration of 50 times the density of a MacFarland nephelometer No. 1 standard. Sufficient antigen was obtained from one production lot to hyperimmunize at least 3 rabbits plus amounts used subsequently as testing antigens. In addition, hyperimmune antiserums were prepared against two commercial PPLO antigens—American Scientific Laboratories (ASL) and University of Connecticut (Storrs)—using the undiluted antigen as purchased.

Apparently healthy rabbits, weighing 4 to 6 lb., were each given a total of at least six 1-ml. injections intraperitoneally or intravenously over a period of 18 to 21 days. Before injection, the serum from each rabbit was checked for negative agglutination against all of the antigens used in this study. Approximately ten days after the last injection of antigen, the rabbits were exsanguinated. The serum was harvested aseptically, dispensed in small amounts, and stored at 4 C.

Agglutination and Cross-Agglutination Tests.—Stock antigens were prepared by diluting the packed cells to a density of the No. 4 MacFarland nephelometer tube and were preserved with 0.3 per cent formalin.

All serum titers and cross-agglutination titers were determined by the tube agglutination test. Serums were diluted with buffered saline solution by twofold dilutions beginning at 1:5. To 0.5-ml. amounts of the serum dilutions was added 0.5 ml. of antigen. The tubes were shaken and incubated in a water bath at 37 C. Final readings were made at 48 hours. Each test was appropriately controlled, and all tests were replicated.

Agglutinin Absorption.—The serums were subjected to the agglutination absorption technique as follows: The serum to be absorbed was diluted 1:5 with buffered saline solution. An equal volume of antigen was added and absorption carried out at 37 C. (water bath) for two hours followed by refrigeration at 4 C. overnight. The tubes were centrifuged and the supernate drawn off. A second absorption was usually necessary.

Hemagglutination and Hemagglutination Inhibition.—Hemagglutination (HA) activity against chicken erythrocytes and the hemagglutination inhibition (HI) and cross inhibition of the rabbit serums were determined by the procedures described by Gianforte *et al.*,[8] using the stock antigens described previously.

Carbohydrate Fermentation.—The carbohydrates to be tested were prepared in a final concentration of 1 per cent in phenol red broth base (Difco) which was enriched with 1 per cent PPLO serum fraction. The isolates were tested for their ability to utilize the following carbohydrates: glucose, sucrose, maltose, lactose, mannitol, levulose, mannose, trehalose, dulcitol, galactose, starch, dextrin, xylose, and salicin. Cultures were incubated at 37 C. and examined for acid production twice daily for seven days.

Colonial Characteristics.—The stock broth cultures were routinely transferred at weekly intervals. At this time each culture was additionally checked by plating on PPLO agar (Difco) supplemented with 1 per cent PPLO serum fraction. The plates were incubated at 37 C. for one week, during which time each culture was periodically examined microscopically for colonial characteristics and morphology, the latter by the agar fixation method of Klieneberger,[14] using Bouin's fluid and Giemsa stain. The colonies were also stained by the Dienes method[4] and examined.

Pathogenicity.—TURKEY POULT.—The inability of three of the isolates (54-537, HPR-5, HPR-15) to produce sinusitis or air sac lesions or both and a serologic titer in turkey poults had been previously determined. All of the remaining isolates (table 1), including the strains subsequently received from Adler, were similarly tested during this study as follows: 0.5-ml. amounts of the broth culture were inoculated into the right infraorbital sinus of each of 2 serologically negative turkey poults. In addition, 0.5 ml. of the culture was introduced intratracheally in 1 of the poults. The birds were isolated and examined over a period of

TABLE 1—Source of Cultures

Isolate	Source	Method of isolation	Culture received in	Remarks
54-537	Ga.–chicken	artificial culture medium	.....	isolated from live bird by tracheal swab
HPR-15	Ga.–chicken	artificial culture medium	.....	trachea
DIVA	Ga.–chicken	*in ovo*-yolk sac	.....	tracheal exudate
DIVBp	Ga.–chicken	*in ovo*-yolk sac	.....	tracheal exudate
CRDA	Mass.–chicken	*in ovo*-yolk sac	amnioallantoic fluid	tracheal exudate
NY	N.Y.–chicken	*in ovo*-yolk sac	frozen yolk	tracheal exudate received from Dr. Markham
HPR-5	Pa.–chicken	artificial culture medium	culture medium	isolated from live bird by tracheal swab
W	Va.–chicken	*in ovo*-yolk sac	culture medium	Winchester strain
VR	Conn.–chicken	*in ovo*-yolk sac	culture medium	received from the late Dr. E. P. Johnson—identified as Mass. A 5969
S-6	Calif.–turkey	artificial culture medium	culture medium	isolated from brain of turkey
Sv	Calif.–turkey	artificial culture medium	culture medium	sinus
TU	Calif.–chicken	artificial culture medium	culture medium	turbinate
Sa	Calif.–turkey	artificial culture medium	culture medium	trachea
O	Calif.–turkey	artificial culture medium	culture medium	pericardium
N	Calif.–poult	artificial culture medium	culture medium	air sac, day-old poult

†Sorvall Refrigerated Centrifuge, manufactured by Ivan Sorvall, Inc., Norwalk, Conn.

30 days for clinical evidence of respiratory disease. Blood samples were taken from each bird; they were then killed and examined for lesions. The sinuses were cultured for PPLO and the serums were tested for homologous agglutination titer. Each serum was also tested for titer against two commercial PPLO antigens (ASL and Storrs).

CHICKEN EMBRYO.—As in the case of the turkey poults, isolates 54-537, HPR-5 and HPR-15 had previously been shown not to possess pathogenicity for the chicken embryo. The remaining cultures were examined for *in ovo* pathogenicity by inoculating 0.2 ml. of culture into each of 5 seven-day chicken embryos via the yolk sac route. The eggs were incubated at 37 C. for one week. They were candled daily and all deaths prior to 48 hours incubation were considered as due to mechanical injury or contamination. The yolks from embryos dying between the third and seventh day were harvested and used for additional studies. At the same time, they were cultured for PPLO. No isolate was considered nonpathogenic for the chicken embryo until at least five blind passages had been performed and the PPLO were again isolated from the harvested yolk. Lesions encountered were typical of those which have been previously described in the literature.

RESULTS

The homologous and cross-agglutination titers are recorded (table 2); homologous titers ranged from 1:160 to 1:1,280. Cross agglutination occurred to some degree with most of the isolates and in several instances with titers close to their homologous titer. There were three groupings which contained two or more isolates, *viz.*, 54-537 and HPR-5; CRDA, DIVBp, DIVA and TU; and VR, W, Sv, and S-6. The five remaining isolates did not cross-react to any great degree. Although the VR, W, Sv, and S-6 isolates are apparently closely related, S-6 appears to be distinctive in that it was not agglutinated by any other antiserums except Sv, VR, and W.

The results with the agglutinin absorption technique correlated closely with the results obtained by cross agglutination, indicating that many of these isolates possessed common antigenic components. In some instances absorption was nearly complete, thus confirming the serologic relationship of the isolates within the group (54-537 and HPR-5; DIVBp, CRDA, DIVA, and TU; and VR, W, Sv, and S-6).

The HA reactions are recorded (table 3). Only a few of the isolates possessed the ability to agglutinate chicken erythrocytes; titers of 1:128 or higher were obtained with the VR, W, Sv, and S-6 isolates. The HI and cross-HI results with these strains indicated the same close antigenic relationship as seen in the cross-agglutination tests.

The general activity of the isolates regarding carbohydrate fermentations are summarized (table 3). All strains in serotypes A, C, D, and F fermented glucose, sucrose, maltose, levulose, trehalose, galactose, starch, dextrin, and mannose (except Sa) but did not ferment lactose, duleitol, and salicin. Xylose was fermented by W, VR, Sv, and S-6, but was not fermented by DIVBp, CRDA, and NY. On the other hand, DIVA, TU, and Sa produced only slight amounts of acid in xylose. The W, VR, Sv, and S-6 strains produced some (slight) acid in mannitol after seven days' incubation, whereas none of the other strains fermented this carbohydrate. The 54-537,

TABLE 2—Homologous and Cross Agglutination Titers of Various Isolates of Avian Pleuropneumonia-Like Organisms

Antiserums	Antigens														
	54-537	HPR-5	TU	DIVA	CRDA	DIVBp	HPR-15	NY	Sa	S-6	Sv	VR	W	O	N
54-537	160	160	20	—	—	—	20	40	—	—	—	—	—	—	—
HPR-5	160	160	20	—	—	—	40	40	—	—	—	—	—	—	—
TU	40	40	640	640	160	160	40	40	20	—	40	40	40	—	—
DIVA	40	40	640	640	160	20	40	40	20	—	40	20	—	10	—
CRDA	40	40	320	320	640	160	20	40	40	—	—	20	20	—	—
DIVBp	80	80	320	640	80	1,280	40	20	—	—	40	—	40	—	—
HPR-15	—	—	—	—	—	—	320	—	—	—	—	—	—	—	—
NY	80	80	40	20	40	40	80	1,280	80	—	10	—	40	—	—
Sa	10	10	40	20	—	20	80	20	1,280	—	—	—	—	—	—
S-6	—	—	—	40	40	20	40	40	20	320	320	320	320	—	10
Sv	—	—	—	40	—	20	20	40	—	160	1,280	160	320	—	—
VR	—	—	—	—	—	—	—	—	—	160	160	320	40	—	—
W	—	—	—	20	20	20	20	20	—	320	320	40	1,280	—	—
O	—	—	—	20	20	20	—	20	—	—	—	20	—	320	—
N	—	—	—	—	—	—	10	—	—	20	—	—	20	—	1,280

Underscored numbers indicate cross reaction; — = No agglutination at 1:10 dilution (tube method).

TABLE 3—Serotypes of Avian Pleuropneumonia-Like Organisms

Serotype	Isolates tested	Culture characteristics		Activity in carbohydrates	HA chicken erythrocyte	Pathogenicity			
						Poult		Chick embryo	
		Growth	Colony*			Lesions	Titer	Lesions	Mortality
A	W	Slow	SRU	+	+	+	+	+	+
	VR†	Slow	SRU	+	+	−	+	−	−
	S-6**	Slow	SRU	+	+	+	+	+	+
	Sv‡	Slow	SRU	+	+	+	+	+	+
B	54-537	Rapid	LFE	−	−	−	−	−	−
	HPR-5	Rapid	LFE	−	−	−	−	−	−
C	TU‡	Rapid	LFE	+	−	−	−	−	−
	DIVA	Rapid	LFE	+	−	−	−	−	−
	(DIVA-yolk)§					(+)	(+)	(+)	(+)
	DIVBp	Rapid	LFE	+	−	−	−	−	−
	(DIVBp-yolk)§					(+)	(+)	(+)	(+)
	CRDA	Rapid	LFE	+	−	−	−	−	−
	(CRDA-yolk)§					(+)	(+)	(+)	(+)
D	NY	Rapid	LFE	+	−	−	−	−	−
	(NY-yolk)§					(+)	(+)	(+)	(+)
E	HPR-15	Rapid	LFE	−	−	−	−	−	−
F	Sa**	Rapid	LFE	+	−	−	−	−	−
G	O**	Rapid	LFE	−	−	−	−	−	−
H	N**	Rapid	LFE	−	−	−	−	−	−

* SRU = small, granular, raised colonies, deeply and uniformly stained by the Dienes method;[4] LFE = large, flat colonies with raised centers, dark stained center, lighter stained lacey periphery. † This strain has been maintained in well over 500 passages and is no longer capable of producing lesions. ** Designated as antigenic types by Adler *et al.*[4] ‡ Indicated by Adler (personal communication) as representing types S-6 (Sv) and C (TU). § Pathogenicity demonstrated with stock egg-yolk cultures only.

HPR-5, HPR-15, O, and N isolates failed to ferment any of the carbohydrates tested.

All of the isolates required serum or PPLO serum fraction in the mediums for growth. All of the isolates grew in the liquid and solid mediums tested; however, it was consistently observed that VR, W, Sv, and S-6 required three to five days' incubation for optimum turbidity and colony size as compared to one to three days for the other isolates.

All colonies were microscopic in size and were best examined when magnified 30 to 100 times with a wide-field microscope. Attempts to select cultures which would produce either small or large colonies more uniformly were negative. In several instances, the small and large colonies were picked and cultured separately for several passages and each culture was then checked against homologous and heterologous antiserums. No differences were observed. A similar technique was carried out after first plating young (72-hr.) cultures and old (6-day) cultures of the isolates. In no instances were subcultures obtained which did not have titers in agreement with the stock antigen prepared from the respective isolate.

Two major types of colony and cell morphology occurred. The VR, W, Sv, and S-6 isolates consistently produced small (up to 0.1 mm. in diameter), highly refractile, granular, raised colonies. On staining with the Dienes technique they appeared as small, dense, deeply stained masses. The organisms appeared as small coccobacilliform elements. This was in contrast to the other isolates, where the colonies were larger (0.3–0.5 mm.), flat, and had raised centers. After staining, the colonies showed a dense, dark center and a lighter stained, lacey periphery (so-called "fried-egg" appearance). The organisms in young (24- to 48-hour) cultures showed some small coccoid elements, but so-called ring forms and large vacuoles predominated.

Table 3 summarizes the results obtained in the pathogenicity studies. Only one serologic group (serotype A) had isolates which produced typical evidence of pathogenicity including serologic titers. Isolate VR, which was well over the 500th culture passage when tested, was no longer pathogenic; however, it did produce a detectable titer in the poults. The W, Sv, and S-6 isolates produced sinusitis in poults and typical lesions and death patterns in chicken embryos. The results with four of the isolates are somewhat confusing; DIVA, DIVBp, CRDA, and NY, when grown in artificial mediums, did not produce lesions or titers in poults or a typical *in ovo* pattern. However, when the original stock egg-yolk cul-

tures were passaged in chicken embryos opposite results (as shown in parentheses in table 3) were obtained. Furthermore, this same yolk material when inoculated into turkey poults produced a slight sinusitis and at least a "suspicious" titer when tested with S-6 antigen. None of the remaining serotypes exhibited any evidence of pathogenicity.

DISCUSSION

The results of cross-agglutination and agglutinin absorption reactions indicate that the avian PPLO are antigenically related. The nine isolates from the author's collection and the six strains received from Adler could be grouped into eight serotypes, designated A, B, C, D, E, F, G, and H (table 3). Adler's strains S-6 and Sv, which he classed as S-6 type, agreed serologically with VR and W in serotype A; his TU strain cross-reacted with members of serotype C and Sa. Strains O and N had no antigenic counterpart in any of the remaining serotypes (B, D, or E).

The biologic and pathogenic characteristics of the serotypes have been summarized (table 3). The serotypes exhibited two major classifications regarding culture and pathogenic characteristics. Serotype A contained strains (W, VR, Sv, S-6) which were slow-growing as contrasted to the rapid growth of the isolates in the remaining types. Furthermore, they grew as small, granular, raised, refractile colonies which stained deeply and uniformly by the Dienes method, and morphologically resembled the coccobacilliform bodies of Nelson.[18] Conversely, the colonies produced by serotypes B through H were larger, less refractile, raised in the center and flat in the periphery. When stained by the Dienes method they showed a dense, dark-stained center and a lacey, lighter stained periphery. Morphologically, ring forms predominated. The isolates in type A agglutinated chicken erythrocytes with hemagglutinating titers of 1:128 or higher. None of the other serotypes contained isolates with HA activity. Finally, the type A isolates exhibited pathologic characteristics in turkey poults and chicken embryos in contrast to the negative findings in the other serotypes. Thus, serotype A contained only pathogenic strains, whereas nonpathogenic strains made up the remaining serotypes.

The fact that there may be eight or more serotypes among the avian PPLO is not necessarily of much consequence. What is of considerable significance is the observation that the pathogenic strains from both collections fell into one serotype which was serologically distinguishable from the other types of avian PPLO under study. Jungherr *et al.*[12] and Gianforte *et al.*[8] reported on pathogenicity and serologic studies with seven strains of PPLO isolated from cases of CRD and IS from different avian species and geographic areas. They selected only pathogenic strains and did not include isolates from nondiseased birds. The present observations on the antigenic homogenicity of pathogenic PPLO, that is, the existence of one pathogenic serotype, corroborates their conclusions. They indicated that "variations in pathogenicity of avian PPLO were due to innate characters and not to type differences . . . apparently the PPLO of CRD exist as one species."

Based on serologic, culture, and pathogenic characterizations, it would appear that the S-6 strain should be considered as the type strain of the pathogenic PPLO. Observations in this study corroborate those reported by Adler *et al.*[4] and indicate that S-6 cross-reacts to a high degree with all of the so-called pathogenic strains thus far tested from various sources. The morphologic, culture, and biochemical characteristics are likewise in agreement with other pathogenic strains. Furthermore, the results obtained with the two commercial antigens (table 4) and the development of titers only in turkey poults inoculated with pathogenic PPLO (table 3) substantiates the efficacy of agglutination antigens made from strains of the S-6 type for the detection of CRD and IS infections. This points up the desirability of careful selection of strains to be used for the production of agglutination test antigens.

Jungherr *et al.*,[11] in applying the HI test for the diagnosis of CRD, indicated that HA activity might be considered as a characteristic of the CRD strain. White *et al.*[21] and Fahey [7] noticed that both turkey sinusitis and chicken CRD strains cross-reacted on HI tests and suggested the value of the test in detecting CRD and IS infection. Adler *et al.*[3] found that the pathogenic strain (S-6) agglutinated washed chicken blood cells, whereas they were unsuccessful in demonstrating HA activity from the other (nonpathogenic) strains under study. In the

present study, only the pathogenic strains possessed the ability to agglutinate chicken erythrocytes, and cross hemagglutination-inhibition occurred only with serums produced from strains in serotype A. These results lend credence to the efficacy of the HI test as a diagnostic tool in CRD and IS. Furthermore, HA activity would appear to be a significant characteristic of pathogenic avian PPLO. Information on the rapidity with which agglutinating and HI titers develop in infected birds, the persistence of titers, and the duration of infection was not determined in these investigations.

The fermentation of carbohydrates has been regarded as characteristic of the agent of CRD and IS by Grumbles *et al.*,[9] Gianforte *et al.*,[8] and Adler *et al.*[3,4] In general, the fermentation reactions observed in this study are in agreement with their results; however, there was no complete uniformity in the carbohydrates tested. The pathogenic strains fermented glucose, sucrose, maltose, levulose, mannose, trehalose, galactose, starch, dextrin, and xylose. They did not ferment lactose, dulcitol, and salicin. The results with mannitol are inconclusive. It should be noticed that mannitol was negative in the reports cited. Gianforte and Adler reported no fermentation of galactose and xylose; however, in an earlier paper, Adler *et al.*[3] reported that galactose was fermented. The results with the fermenting nonpathogenic strains indicated that mannitol was not fermented, and four strains (DIVBp, CRDA, NY, and Sa) were negative or only slightly positive in xylose. Adler *et al.*[3] cited delayed (48 hr. or longer) fermentation of sucrose as a possible characteristic of pathogenic strains. This feature could not be corroborated in the present study, since a number of the nonpathogenic strains failed to ferment sucrose for as long as 72 to 96 hours. There were four serotypes which failed to ferment carbohydrates. Significantly, none of the pathogenic strains fell into any of these groups. This feature has diagnostic significance; however, the fermentation of certain carbohydrates for the differentiation of pathogenic and nonpathogenic isolates needs further study.

The lack of pathogenicity observed with the artificial culture isolates of DIVA, DIVBp, CRDA, and NY was viewed with some concern; the egg-yolk cultures of these strains had always been pathogenic for turkeys and chick embryos. These strains were, in fact, the stock strains of pathogenic PPLO used by the author during 1952 to 1956 in various studies on the characterization and pathogenicity of the PPLO of CRD. At that time, culture isolations of these strains made from the egg-yolk material yielded PPLO which morphologically and pathologically resembled the characteristics as described herein for serotype A. When the present study was instigated, it was planned to re-isolate the PPLO from the original stock egg-yolk cultures in artificial culture mediums. This was done; however, it was soon apparent that the PPLO which had been isolated were not pathogenic, possessed no HA activity, and did not resemble the original PPLO in colony and morphologic characteristics. Repeated attempts at culturing the egg-yolk material failed to yield pathogenic PPLO, yet injection of this same egg-yolk material into poults and chicken embryos resulted in lesions, titers, and typical *in ovo* patterns for pathogenic PPLO (*see* results in parentheses, table 3). Several attempts at culturing the sinuses of inoculated poults and the dead embryos also failed to yield pathogenic PPLO, but did yield the nonpathogenic types recorded in table 3 for these four strains. The original artificial culture isolates which had been lyophilized and stored at −60 C. for several years were then subcultured, but unfortunately were nonviable.

TABLE 4—Agglutination Reactions of Two Commercial Antigens Against Rabbit Antiserums Injected with Isolates of Avian Pleuropneumonia-Like Organisms

Antiserums	Antigens	
	ASL	Storrs
54-537	—	—
HPR-5	—	—
TU	—	—
DIVA	—	—
CRDA	—	—
DIVBp		
HPR-15	—	—
NY	—	—
Sa	—	—
O	—	—
N	—	—
W	160	160
VR	80	80
Sv	320	160
S-6		

It is the author's opinion that the stock egg-yolk cultures of DIVA, DIVBp, CRDA, and NY had at some time during serial egg passage become contaminated with a nonpathogenic, egg-transmitted PPLO. Since

the nonpathogenic strain grows so readily and rapidly in artificial culture mediums, it consistently overgrew the pathogenic PPLO whenever isolations were made in artificial culture mediums.

SUMMARY AND CONCLUSIONS

Evidence is presented which indicates that avian pleuropneumonia-like organisms (PPLO) may be grouped into various serotypes. Of significance, however, is the finding that strains capable of producing lesions and serologic titers in turkey poults, *i.e.*, pathogenic PPLO, fell into just one serotype and none of the nonpathogenic PPLO reacted with this serotype. The pathogenic serotype could be readily differentiated from the other groups of nonpathogenic PPLO by agglutination, hemagglutination, and hemagglutination-inhibition reactions. Only the strains in the pathogenic serotype possessed the ability to hemagglutinate chicken erythrocytes.

The strains in the pathogenic serotype were consistently slower growing, yielding forms apparently similar to Nelson's coccobacilliform bodies, and produced small, refractile, granular, raised, and uniformly stained colonies. This was in contrast to so-called "ring forms" observed in nonpathogenic PPLO. Here the colonies were larger, with raised centers, which on staining showed a dark center and lacey periphery.

The use of agglutinating antigens for the detection of chronic respiratory disease and infectious sinusitis in chicken and turkey flocks is feasible. Pathogenic strains were not agglutinated by hyperimmune rabbit serums produced from nonpathogenic strains. Furthermore, the introduction of pathogenic strains into turkey poults yielded readily detectable serologic titers, whereas the inoculation of nonpathogenic strains failed to elicit detectable titers, either to their homologous antigens or the antigens of pathogenic strains.

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XUM

Infection of an Isolated Intestinal Loop by Newcastle Disease Virus

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IT HAS BEEN demonstrated that after infection of a susceptible chicken with Newcastle disease virus (NDV), a part of the developmental cycle of the virus occurs in the intestinal wall.[2-4] It has also been shown that from the primary locus of multiplication in the gut-wall the virus invades the blood stream, multiplies in the spleen, and returns to the intestine. This cycle is followed by the excretion of large amounts of virus into the lumen of the gut and feces.

This sequence of events could be demonstrated in a clearer manner if the fate of the virus in the intestinal tract were not disturbed by the continuous flow of digested food materials through it. Such an opportunity was offered by the method of Newman and Taylor,[5] which permits resection of the lower intestinal tract and connection of an isolated loop of it to the outside of the body by two nylon cannulas; The blood and nerve supply of the loop are not interrupted. The operation was described and illustrated in detail by Newman and Taylor.[5] After incising the right lateral abdominal wall, the segment of small intestine which has the yolk stalk attached (corresponding to ileum in mammals) is withdrawn and a 5- to 7-cm. portion, supplied by the radial mesenteric blood vessels nearest the yolk stalk, is resected. The continuity of the interrupted intestine is re-established with a side-to-side anastomosis, care being taken to have isoperistaltic connection. The ends of the isolated portion are sutured over nylon cannulas, one end of which passes out through each extremity of the abdominal incision when it is sutured. A schematic diagram of the arrangement is presented (fig. 1).

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The authors are indebted to Dr. Vincent Groupé, of the Institute of Microbiology, who made it possible to perform these experiments.

MATERIALS AND METHODS

A 15-week-old chicken, susceptible to Newcastle disease, was subjected to the technique previously described. Six days after surgery, when the wound was completely healed and the chicken was in good health, the intestinal loop was cleaned by passing sterile tissue culture nutrient medium [1] through it. The loop was then filled with 1.5 ml. of a suspension of NDV, strain B1,* containing 10^5 embryo infectious doses—50 per cent ($e.i.d._{50}$/ml.). At intervals of 5, 10, 30, 60, and 120 minutes, 0.1- to 0.2-ml. samples of fluid were withdrawn from the loop by means of a syringe equipped with a blunt needle. During these two hours, the chicken was held immobile, lying on its side with the nylon fistulas exposed to manipulation. The samples withdrawn were immediately diluted 1:5 or 1:10 into sterile nutrient medium. Two hours after introducing the virus, the loop was rinsed twice with 4.0 ml. of the nutrient medium. This was done by letting the medium drain from a pipette into one end of the loop and withdrawing it at the same rate from the other end with a syringe. Sometimes a slight positive pressure, supplied by a rubber bulb, was applied to the pipette to force the fluid through the loop. To prevent leakage of medium where the pipette entered the fistula, a ½-inch long rubber tube was attached to the fistula, and the tip of the pipette was firmly pressed into the free end of the tube. When the rinsing was completed, about 0.5 ml. of the medium was left in the loop, the two openings were plugged with cotton, and the chicken was put in its cage.

The intestinal loop was flushed at intervals of one to two hours for 12 hours, using this procedure. After 12 hours, the loop was flushed and the samples were collected at 12- to 24-hour intervals for six days. Each time, 4 ml. of medium was used; all of this fluid was collected into small test tubes, plugged with rubber stoppers, taped, and frozen in dry-ice acetone. Before flushing the loop, gelatinous excretions blocking the passage were mechanically removed by means of a miniature cotton swab.

In addition to sampling the medium from the loop, citrated blood samples were taken by cardiac puncture at similar intervals. The feces were collected on paper laid under the grid of the cage. Fluid samples (rinsing fluid and blood) were frozen at −80 C., feces at −20 C., and all were stored at −20 C. until titration. The presence and concentration of the virus in the collected samples

* Obtained from Johns Hopkins University, Baltimore, Md., through the courtesy of Dr. F. B. Bang.

was determined by titration in the allantoic cavity of 10-day chicken embryos. Three to four eggs were used for each five- or tenfold dilution. All materials to be titrated were diluted in saline solution containing 500 units of penicillin and 500 Gm. of streptomycin per milliliter. Feces were weighed, diluted 1:4 in diluent, and homogenized in a Waring blendor; the suspension was centrifuged at 1,600 R.C.F.* for 15 minutes and the supernatant fluid used for titrations. Virus concentration was calculated according to the Reed and Muench formula [6] and expressed as e.i.d.$_{50}$ per gram or milliliter of material.

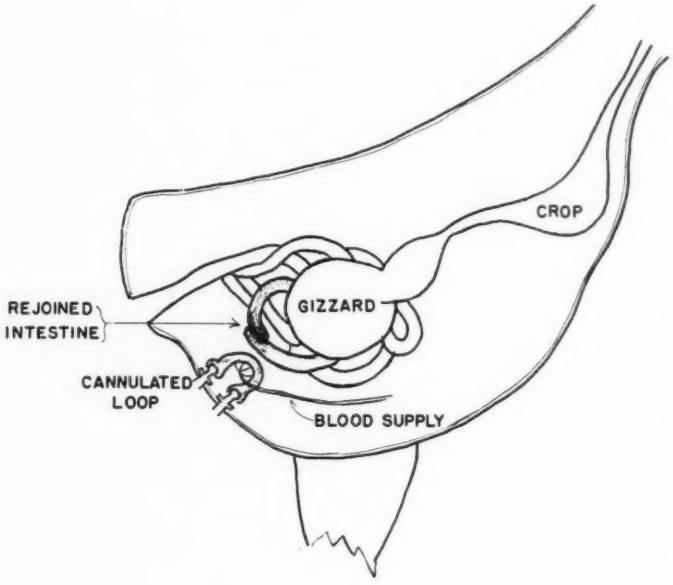

Fig. 1—Schematic drawing of the cannulated intestinal loop in relation to the operated intestine.

RESULTS AND DISCUSSION

Two similar experiments were performed with essentially identical results. The results of one of these experiments are summarized graphically (fig. 2). After introduction of the virus into the loop, there was a gradual adsorption of about 70.0 to 90.0 per cent of the virus within one hour (curve L, fig. 2). When the loop was rinsed after two hours, 99.9 per cent of the virus was removed. The residual 0.1 per cent of the virus (3 to 30 e.i.d.$_{50}$/ml.) continued to be found in the rinsing fluid for at least 14 hours, even after nine successive rinsings. The increase in the concentration of the virus in the loop above this level became apparent in the 22-hour sample. At 34 hours after introduction of the virus, the concentration in the loop reached 10^5 e.i.d.$_{50}$ per milliliter. The production of the virus continued until the third day. On days 4 and 5, there was a considerable drop in the quantity of virus found in the loop and no virus was found there on days 6 and 7. However, a small amount was detected in the homogenate of this tissue when removed at death (day 7).

No virus was found in blood samples taken two, four, seven, 13, and 22 hours after infection (curve B, fig. 2). The first evidence of viremia was found at 34 hours. Maximum viremia persisted from postinfection day 2 to 4. On day 5, there was little virus in the circulating blood (30 e.i.d.$_{50}$/ml.) and no virus on days 6 and 7. Hemagglutination-inhibiting antibodies appeared on day 6.

Excretion of the virus in the feces began only after three days (curve F, fig. 2), reached the maximum on days 5 and 6, and diminished on day 7, when the bird was killed.

These results indicate that after infection of the intestinal loop there is a local multi-

* Relative centrifugal force.

plication of the virus, as evidenced by the shedding of the virus into the lumen of the loop after 22 hours or more. This local increase is followed by invasion of the blood stream after 34 hours and a viremia of three to four days duration; reinfection of the main intestinal tract is indicated by the excretion of NDV in feces, commencing three days after infection. With the appearance of antibodies in the circulatory system, the virus disappears from the blood but contiues to be excreted in the feces.

These findings constitute proof of the previously proposed hypothesis [4] that the infection of the intestinal tract may proceed in a two-step fashion; namely, that after primary local development of the virus in some part of the lower intestine, the virus spreads by way of the blood system to other organs and other parts of the lower intestinal tract, multiplies there in large quantities, and is finally excreted in feces. A point which has not been sufficiently clarified in this experiment is the time of the first appearance of the virus in the tissue of the intestinal loop. According to the evidence obtained elsewhere,[4] the virus could have started multiplying in the cells lining the loop about 12 hours after infection. In the system employed, the proof of this contention would require infection of a large number of chickens with surgically isolated intestinal loops and the removal, homogenization, and titration of such homogenates at various intervals after infection.

In spite of successive rinsing of the loop with fresh medium, some residual virus was present there throughout the first 14 hours after infection. There probably were some hidden folds of the villi from which the virus would not be completely removed by the procedure employed. To show that the dilution procedure itself was sufficient to remove the virus completely, the following experiment was performed: 10^5 e.i.d.$_{50}$ of NDV, strain B1, was introduced in a volume of 1.5 ml. into a U tube made of rubber tubing with nylon cannula ends and kept in an incubator at 38 C. At intervals the tube was flushed with sterile medium in a manner identical with the procedure employed in the case of the chicken. These fluids were collected, frozen, stored, and titrated in the same way as the samples obtained from the infected chicken. The results show (curve C, fig. 2) that after no

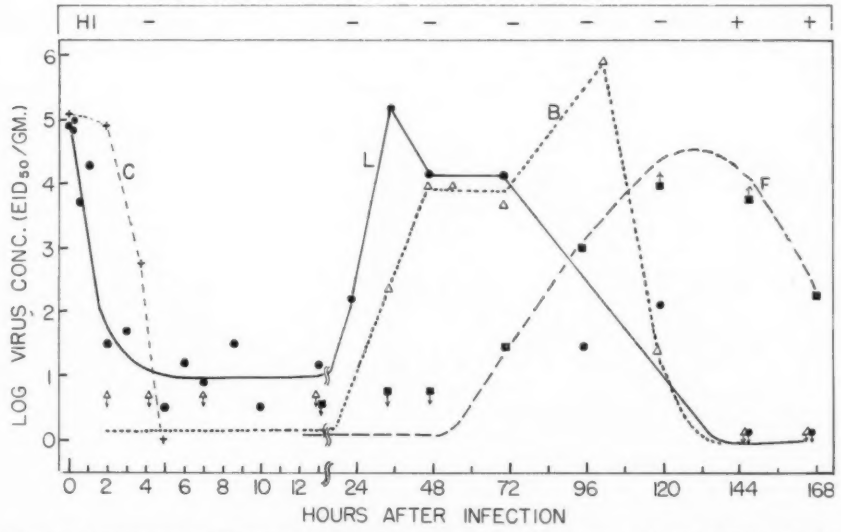

Fig. 2—Appearance of Newcastle disease virus in the intestinal loop (L), the blood (B), and the feces (F) after infection of the intestinal loop. Curve C shows the disappearance of the virus from a rubber loop, due to washing. Downward arrows indicate that no virus was found at the lowest dilution tested, while upward arrows show that the concentration of the virus was higher than indicated.

change in concentration of the virus during the two hours of holding time, the virus disappeared completely after the 4th change of medium. In spite of the great dissimilarity between the rubber tubing with its smooth surface and the intestinal mucosa with its complicated system of villi, one can be sure that nine successive flushings of the intestine should at least theoretically be sufficient to remove the virus entirely. Actually, some residual virus was found in all these nine washing fluids, and one may therefore envisage a gradual release of the adsorbed virus from the nooks between the villi, or from the nonspecific virus-inhibiting mucus produced by the mucous membrane of the intestinal loop.

SUMMARY

Newcastle disease virus introduced into a surgically isolated intestinal loop of a susceptible chicken multiplied locally, commencing about 20 hours after introduction. The virus appeared in the blood after the concentration in the intestinal loop had reached its maximum. Following a period of viremia, the virus was found in fecal excretions three to seven days postinfection, when the bird was killed. With the appearance of antibodies on day 6, the virus disappeared from the intestinal loop and from the blood, but was still found in feces for at least two additional days.

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Comparison of the Pathogenesis of Newcastle Disease Virus and Influenza B Virus in Chicken Embryos

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CORTISONE has been shown to influence the susceptibility of many host animals to infectious disease [9, 10, 12] and to modify the inflammatory response.[3, 7, 11]

Kilbourne and Tateno,[6] working with the Lee strain of influenza B virus, described both an increase and a prolongation of virus production in tissue cultures of chicken chorioallantoic membrane to which small quantities of cortisone had been added. The increase in virus production induced by cortisone might have resulted, the authors speculated, from multiplication of virus in cells which survived longer in treated cultures than in the control cultures. Subsequent work by Kilbourne [5] with the same virus in the chicken embryo demonstrated a prolongation of embryo survival time and a suppression of the inflammatory response in the chorioallantoic membrane of cortisone-treated embryos.

In our work, an attempt was made to study the effects of cortisone on Newcastle disease virus (NDV) and influenza B virus infections in embryos and to investigate the mechanisms involved.

MATERIALS AND METHODS

The Texas-GB-1948 strain of NDV and the Lee strain of influenza B were used. Cortisone was employed as a suspension of hydrocortisone acetate * in sodium chloride solution (25 mg./ml.). All chicken embryos were 10 days old at the time of inoculation of virus into the allantoic chamber.

The effect of cortisone on the mean death time (MDT)[4] of virus-infected embryos was determined. Dosage of cortisone was varied from 0.01 mg. to 0.1 mg. and was administered from 24 hours before to 24 hours after inoculation with virus. Dosage of virus ranged from $10^{2.0}$ to $10^{6.0}$ l.d.$_{50}$ of NDV for the embryo and $10^{2.5}$ to $10^{6.5}$ l.d.$_{50}$ of influenza virus for the embryo. Twenty-four eggs were used in each test group. Eggs were candled every eight hours and deaths of the embryos recorded; the MDT was then calculated for each group.[4]

In order to determine the effects of cortisone on the rate of viral multiplication and on the final viral yields, groups of 8 eggs were inoculated with $10^{6.0}$ l.d.$_{50}$ of the Texas-GB strain; one hour after infection, half of the embryos were treated with 0.10 mg. of cortisone. At 12, 24, and 36 hours after virus inoculation and at death, groups of cortisone-treated and control eggs were removed for harvest. Allantoic fluids and chorioallantoic membranes were harvested and pooled in each group. The virus content of the suspensions was determined by l.d.$_{50}$ titrations in embryonating eggs. To determine the effects of cortisone on the rate of influenza multiplication and on the final yields attained, groups of 8 eggs each were inoculated with $10^{6.5}$ l.d.$_{50}$ of the Lee strain. At 24 hours after infection, half of these embryos were treated each with 0.1 mg. cortisone. Fluids and embryonic tissues were harvested 24, 36, and 48 hours after inoculation and at death. The infective titer of the suspensions was determined in eggs.

To determine the effect of cortisone on the distribution of virus in the embryo, embryos were inoculated with $10^{6.5}$ l.d.$_{50}$ of influenza or with $10^{6.0}$ l.d.$_{50}$ of NDV. Cortisone was administrated 24 hours after the inoculation of influenza B and one hour after the inoculation of NDV. Groups of eggs were kept as untreated virus controls. At specific intervals after infection, fluids and organs were harvested from each group and pooled accordingly. The hemagglutinins of the suspensions were titrated according to the method of Salk.[8]

Influenza inoculum consisted of $10^{6.5}$ l.d.$_{50}$, NDV of $10^{8.0}$ l.d.$_{50}$. Cortisone was administered 24 hours after virus inoculation. Tissues were fixed in formol-Zenker solution and stained with hematoxylin and eosin. Histologic examination was made of chorioallantoic membranes and embryos of infected cortisone-treated, infected-untreated controls, and normal embryos at specific intervals.

RESULTS

The administration of cortisone 24 hours after the inoculation of the Lee strain of influenza B virus resulted in a prolongation of embryo survival time. In no case did cortisone administration have any effect on the survival time of embryos infected with NDV (table 1).

The final yield of virus attained and the rate of multiplication of either virus were

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* Hydrocortone Acetate, produced by Merck & Co., Inc., Rahway, N.J.

TABLE 1—Effect of Time of Cortisone Administration on the Survival Time of Chicken Embryos Infected with Viruses of Newcastle Disease and Influenza

Treatment	Lee $10^{6.5}$ l.d.$_{50}$ survival time*	Yield**	NDV[6] l.d.$_{50}$ survival time	Yield
Cortisone 24 hours prior to virus	49.7	12.1	46	11.9
Cortisone with virus	54.6	10.3	....	12.3
Cortisone 24 hours post virus	71.2	10.3	54	12.3
Virus alone†	54.6	9.9	54	12.1

* Survival time expressed in hours from inoculation to death of embryo.

** Yield = log 2 of hemagglutinins of pooled allantoic fluid harvested at death of embryo.

† Cortisone alone did not cause the death of embryos into which it was inoculated.

unaffected by administration of cortisone.

The distribution of the viruses in the infected embryos differed at several points. Both viruses reached high titers (1:1,280–1:2,560) in the allantoic fluids by 24 hours after infection. The amniotic fluid contained NDV hemagglutinins in a titer of 1:80 by 36 hours after infection, and influenza hemagglutinins in a titer of 1:20 (table 2).

Peak titers of influenza hemagglutinins were present in the chorioallantoic membranes within 24 hours after infection, and by 36 hours they had declined markedly. The NDV hemagglutinins demonstrated in the chorioallantoic membrane within 12 hours continued to increase as the infection progressed and reached a peak of 1:5,120 just before death. Low and possibly nonspecific hemagglutinin titers [3] were detected in the 1:20 and 1:40 dilutions of lung and liver tissues obtained during the course of the infection, whereas NDV hemagglutinins in the liver attained a titer of 1:2,560 and in the lungs, 1:320.

TABLE 2—Distribution of Influenza and Newcastle Disease Virus Hemagglutinins* in Chicken Embryos at Selected Intervals after Allantoic Chamber Inoculation

Hours postinoculation	Amniotic fluid	Allantoic fluid	Chorioallantoic membrane	Lung	Liver**
INFLUENZA VIRUS					
6	0.0†	0.3	1.0	2.5	3.6
12	0.0	2.3	4.3	2.6	3.6
24	1.0	10.0	8.6	3.3	3.6
36–48	1.0	10.0	6.0	3.3	4.0
NEWCASTLE DISEASE VIRUS					
12	0.0	2.5	4.5	0.0	1.5
24	0.5	9.5	9.0	3.0	10.0
36	4.0	10.5	11.0	6.0	10.0

* Titer measured as log 2 of the hemagglutinin.

** Normal hemagglutinin present in the liver may produce a titer of 4.0 or less; the specificity, therefore, should be questioned.

† Each figure is based on three trials (influenza) or two trials (NDV). The underscored replicates did not show a variation of greater than one dilution value.

A moderate inflammatory response was developed in the chorioallantoic membrane 24 hours after the inoculation of the Lee strain. The mesodermal layer was infiltrated with polymorphonuclear leukocytes and round, mononuclear cells. There was moderate fibroblastic proliferation. The ectoderm was hyperplastic. In the endodermal layer there were scattered foci of proliferating epithelial cells, some of which were vacuolated. These changes progressed in degree during the course of the infection and also involved degeneration of capillary endothelium. Administration of cortisone 24 hours after virus inoculation resulted in a marked and consistent depression of the cellular reaction. Both cortisone-treated and control embryos consistently failed to reveal pathologic changes.

The allantoic inoculation of NDV resulted in moderate pathologic changes in the chorioallantoic membrane, which included edema, slight cellular infiltration, and occasional foci of endodermal proliferation. Cortisone effected a reduction in the degree of edema and of cellular reaction. Newcastle disease virus-infected embryos had extensive pathologic changes as early as 28 hours after infection; these lesions were not affected by cortisone treatment. There were massive hemorrhages throughout the embryo by 36 hours. The liver was engorged with blood, most of which was contained within the distended sinusoids. In areas of severe congestion, hepatic cells were degenerated.

DISCUSSION

Cortisone was found to have a definite effect on experimental infection of the chicken embryo with the Lee strain of influenza B virus. However, the course of experimental NDV infection was not altered by cortisone treatment.

It was shown that the administration of cortisone 24 hours after inoculation of influenza virus into the allantoic chamber re-

sulted in prolongation of the embryo survival time, whereas cortisone had no effect on the survival time of embryos infected with NDV. It was thought possible that the prolongation of survival time of influenza-infected embryos could be due to a depression of the virus yields or to a depression of the rate of virus multiplication and, consequently, that it would take longer for the embryos to succumb to the infection. The failure of cortisone to affect the survival time of NDV-infected embryos might then be explained by the lack of a similar effect of cortisone on this virus. Cortisone had no effect on the final yields or on the rate of multiplication of either virus.

Inoculation of the Lee strain into the allantoic chamber produced a local infection confined to the cells of the chorioallantoic membrane. The administration of cortisone 24 hours after virus inoculation and prior to severe damage to the membrane resulted in less severe pathologic changes including both inflammatory response and cellular degeneration. The chorioallantoic membrane is the primary respiratory organ of the developing embryo. Any severe damage to this organ apparently results in the death of the embryo. Cortisone appears to prolong the life of the embryo by suppressing the damage to the chorioallantoic membrane.

The allantoic chamber inoculation of the Texas-GB strain of NDV resulted in a rapid general infection of the entire embryo and its membranes. Pathologic changes were extensive and severe in the body of the embryo, though only slight in the chorioallantoic membrane. Although cortisone partially suppressed the pathologic changes in the membrane, it seemed unlikely that the death of the embryo infected with NDV was due to the relatively mild damage to the membrane. Cortisone failed to decrease the severity of the pathologic changes in the embryo body, possibly because these changes were not primarily the result of an inflammatory reaction.[1] The failure of cortisone to affect the survival time of embryos infected with NDV appears to be due to the generalized nature of this infection.

SUMMARY

The administration of cortisone 24 hours after the inoculation of the Lee strain of influenza B virus into the allantoic chamber of chicken embryos resulted in a prolongation of embryo survival time; it had no similar effect on the course of Newcastle disease virus (NDV) infection. Cortisone did not affect the rate of multiplication or the final yields of either virus. Allantoic chamber inoculation with the Texas-GB-1948 strain resulted in a rapid, general infection with extensive damage to the embryo. Allantoic chamber inoculation with the Lee strain produced an infection largely confined to the cells of the chorioallantoic membrane. The administration of cortisone 24 hours after the inoculation with the Lee strain was found to suppress or reduce the extent of inflammatory response of this membrane. It failed to alter the nature or extent of the pathologic changes induced by NDV in the embryos. The prolongation of survival time of embryos infected with influenza virus appears to have been due to the suppression of cellular damage to the membrane. The failure of cortisone to affect the course of NDV infection of the embryo appears to be due to the fact that the infection becomes generalized.

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The Survival of Foot-and-Mouth Disease Virus in Cured and Uncured Meat

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ANIMALS in certain stages of infection with foot-and-mouth disease virus (FMDV) may escape detection by the usual antemortem or postmortem inspections made at abattoirs. Meat from such animals may inadvertently be included in commercial products. In some countries, the carcasses of animals infected with foot-and-mouth disease (FMD) are purposely used for economic reasons. This situation has prompted many studies on the factors involved in the survival of virus in the tissues of infected animals.

This study assesses the problem in relation to infected beef handled by commercial boning and curing methods.

REVIEW OF LITERATURE

Basic Considerations.—While hanging in the cooler, the carcass of an animal undergoes certain desirable chemical changes in the ripening process. The soluble proteins coagulate and, from the breakdown of glycogen, lactic acid is produced.[19] The meat changes from a slightly alkaline to an acid reaction. *Rigor mortis* is a physical sign that chemical changes are taking place. However, rigor has been demonstrated to set in without any changes in pH and apears to be associated with the disappearance of ATP (adenosine triphosphate) in the muscle tissue.[13, 20] As *rigor mortis* subsides, the muscle fibers relax. The meat becomes more tender due to the action of autolytic enzymes (proteases), which partially break down the more complex meat proteins. Lactic acid brings about the conversion of the collagen of the connective tissue into gelatin.[20] The fats do not change appreciably at refrigerator temperatures. However, at warmer temperatures and in the presence of water, the fats undergo hydrolysis to produce glycerol and fatty acids. In exposed areas where oxygen is available, these products may be further broken down to the lower fatty acids and aldehydes.[19] The pigment in exposed muscle surfaces is oxidized to produce a darker surface color. The rate of progress of these chemical changes is affected by the storage temperature. Quick freezing may temporarily stop the changes, but they resume again when the carcass is thawed.[11, 18] It has been shown that quick frozen meat, when thawed, undergoes a rapid change in pH. In one hour, the pH drops from near the neutral point to values as low as 5.4.[9]

The pH of beef at slaughter is about 7.2 and, an hour later, ranges from 6.5 to 6.8, with some readings as high as 7.2.[13, 20] When a muscle is in full rigor, the pH may range from 5.4 to 6.0.[11] After 48 hours of storage at temperatures slightly above the freezing point, the pH averages 5.6 to 5.8.[2] After about 96 hours of storage, the pH begins to rise as a result of autolysis and the growth of microorganisms.[20] If the pH level rises to 6.4, the keeping quality of the meat may be affected, and there may be suspicion of incipient decomposition.[20] The pH level reached by the meat of normal animals depends on at least two factors: (1) the glycogen content of muscle at the moment of death, and (2) the buffering capacity of the muscle.[2, 11] In ripened meat from normal animals, a pH value lower than 5.3 has not been found, but if the activity of the animal was severe and prolonged prior to slaughter, the pH may be as high as 7.0.[2, 11] Certain disease conditions also tend to prevent lactic acid formation and result in high pH readings.[2] The pH of pork and mutton seldom falls below 5.7, probably because these meats contain less glycogen.[11]

Muscle Tissue.—The inactivation of foot-and-mouth disease virus in the muscles of a carcass is gradually brought about by the chemical changes previously noted. The primary factor is considered to be the production of lactic acid.[10] Prolonged survival of the virus may be expected in meat if the pH value does not fall below 6.2.[9] When acid formation is suspended by quick freezing, the virus may survive for months.[9]

Tissue fluids (pH 5.8), extracted from normal guinea pig muscles at the peak of *rigor mortis* and also from muscles two days after death, inactivated the virus (over 1,000 m.i.d.).[6] Tests with lactic acid solutions showed that the concentration should be 1:10,000 (pH 3.5) or above, since greater dilutions did not inactivate the virus.[7] In tissue culture studies, small fractions of the virus population were able to withstand a change in hydrogen ion concentration for longer periods than the main bulk of the population.[1] Apparently, the inactivation of the virus in meat may be a function of hydrogen ion concentration and

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time and the exact point of extinction would be difficult to state with certainty. Pyl [15] introduced other variables by reporting on the re-alkalization effect and stating that the virus has two pH stability maximums, one at 7.5 to 7.7 and another at 3.0, and two pH stability minimums, 6.0 to 6.5 and 10.0 to 11.0. These findings have not been confirmed.

There have been numerous studies which report histologic lesions in the muscles of infected animals. The distribution of the virus in muscle tissue may not be uniform.[10, 12] Thus, the virus in the muscles may be accounted for by either the direct infection of the tissues or by its presence in the capillary beds and vessels because of viremia.

The gross degenerative changes in muscle tissue occasionally observed in FMD epizootics were produced experimentally in calves and young cattle with type A5 FMDV by Korn and Potel.[12] They reported that virus titers of samples taken from the areas of muscle tissue degeneration usually were higher than those of samples from adjacent muscle tissue. The *biceps femoris* from 1 animal was stored at room temperature for 72 hours and in the grossly involved area of this muscle (pH 6.4 to 6.6), virus was found (titer 10^2).[12]

The early studies on the survival of the virus in meat were accomplished with infected guinea pigs.[6, 10, 13] In these animals, the virus persisted in muscle tissue stored at 2 to 7 C. for as long as seven days.[6] When the animals were not bled out, the virus persisted as long as 31 days.[6] In another study, using similiar storage temperatures, the limit of virus survival was 19 hours.[10] The survival period was reduced to nine hours when the temperature of storage was 17 to 19 C.[10] At −15 C., virus persisted for as long as 21 days (Lavrova, quoted by Zhytenko [24]).

Studies with infected cattle carcasses held at −1 C. failed to demonstrate virus in muscles after storage for 13, 14, 22, 24, and 33 days.[8] Virus was found, however, in cheek and tongue tissue at 33 days. and in meat-wrapping cloths at 40 days.[8] In experimentally infected calves, virus was present in the muscles of 24.5 per cent of the animals up to about 12 hours after slaughter (Travinskii, quoted by Zhytenko [24]).

The studies of Henderson and Brooksby [9] are considered the most important on the subject because the work was done with bovine material handled under abattoir conditions. The test animals were cattle, which are regarded as ideal for tests involving virus of bovine origin. Most other workers used either guinea pigs for donors and test animals, bovine material tested in swine, or swine material tested in suckling mice. In infected beef stored at 4 C., the virus survived in muscle tissue for 24 hours, but it was inactivated by the third day, when the pH was 5.3.[9] Virus survived for months in frozen meat.[9] However, it was reported that it was difficult to demonstrate the virus in quick-frozen meat without resorting to the technique of thawing the meat in a buffered solution.[9]

Studies with infected swine conducted by the British workers [6] failed to demonstrate the virus in pork after one day at usual holding temperatures, and tests were negative with pork held at freezing temperatures and tested on the twelfth day. The virus was found in the bone marrow of these animals. The studies of Wittman [22, 23] indicated that fresh, uncured "bacon" from infected animals contained a considerable amount of virus. When such pork was frozen at −15 C., the virus remained infective for at least 55 days. Frozen pork that was thawed and held at room temperature contained infective virus for at least five days.

The danger of surface contamination of stored meat with virus from freshly slaughtered carcasses was discussed by Zhytenko.[24] He emphasized the long persistence of the virus in certain parts of the carcass, in blood, and on meat-handling equipment. It had been shown by the British workers [6] that virus survived for at least four days in infected blood which was deliberately splashed on the surface of a swine carcass held at 15 C. Even though the virus may be inactivated in muscle tissue, the danger of secondary contamination is ever present in an abattoir that handles infected animals.

Bone Marrow.—Studies have been made which show that the bone marrow does not develop sufficient acidity to inactivate the virus.[6, 8] In the British report,[6] Stockman *et al.* recorded survival of the virus in bone marrow of infected guinea pigs held in cold rooms for as long as 84 days, in beef for 76 days, in chilled pork for 42 days, and in frozen pork for 76 days. They reported the observation that the marrow did not infect swine that were fed the material unless the crushed bone was included. The small injuries to mouth and tongue produced by the bone fragments were apparently important in creating a portal for infection. In the British report,[8] Andrews *et al.* observed survival of the virus in bone marrow for as long as 80 days in bovine carcasses held at about −1 C. In the same report,[8] Galloway showed that alternate freezing and thawing had little effect on virus survival and titer in guinea pig bone marrow.

Lymph Nodes and Blood.—The lymph nodes, and blood clots in large vessels, even though in close proximity to the muscles, do not develop the degree of acidity that is present in the muscle tissue.[9] Viable virus was found in lymph nodes of beef stored at −10 to −20 C. for nearly six months. The pH was as high as 6.5, while in adjacent muscle tissue it was about 5.6.[9] In the same study, the virus was not found in lymph nodes of beef stored at 4 C. for about five and six months. The virus was present in defibrinated blood samples stored at 4 C. for six weeks.[9] Wittman [23] stored pork at 4 C. and found virus in blood clots after 70 days. He also demonstrated the virus in fresh and frozen lymph nodes.

Internal Organs.—Studies with infected guinea pigs showed that the virus persisted in the liver, kidney, and other internal organs.[6] Henderson and Brooksby [9] pointed out that the nonpapillated

pillars of the rumen of cattle are frequently the site of small vesicles that often escape notice. They reported survival of the virus in rumen tissue stored at —20 C. for nearly six months. At the same storage temperature, virus was found in liver for nearly six months and in kidneys for as long as seven weeks. Other studies with bovine liver and spleen held at 4 C. showed that the virus survived for more than 24 hours.[14] After 48 hours, however, the virus was inactivated.

In infected hogs, the virus was found in liver, lung, spleen, kidneys, brain, stomach, and intestines.[23] The virus persisted for long periods when these organs were frozen. At room temperature (21 C.), virus could be demonstrated between four to six days in some of the organs, but by the tenth day, it was present only in the kidneys and blood. The spleen offered little protection for the virus, while the kidneys apparently favored virus survival.[23] Infectious virus was found in the heart blood of a hog for as long as 34 days after slaughter.[6]

Gaugusch and Niemczycka[5] reported on the survival of virus in intestines used for casings. They recommended soaking the casings in 0.5 and 0.25 per cent lactic and tartaric acid solutions to inactivate the virus. Niggli[14] recommended that the surfaces of internal organs be disinfected with 2.0 per cent lactic acid solution.

Waldmann *et al.*[21] used chemical and physical concentration methods to demonstrate the persistence of virus in the serum and urine of immune animals. The virus was found in the urine of normal appearing immune animals for as long as 246 days after infection.

Curing Mixtures.—The second progress report of the British Commission for Research on FMD[6] contains records of experiments on the survival of the virus in various solid and liquid salt mixtures commonly used in curing and pickling meat. The tests were conducted with infected guinea pig foot pads, whole feet, and epithelial tisues from generalized lesions. The solutions and mixtures contained varying proportions and combinations of sodium chloride, saltpeter, sodium bicarbonate, sugar, and boric acid. The conclusion was that such mixtures had little effect on the virus. Infectivity was retained in four tests as long as seven to 11 days, but was lost by the eighteenth. In other tests, the virus survived as long as 49 days. A pickling solution containing vinegar inactivated the virus as a result of lowered pH. Survival of the virus for more than two years in pickling solutions containing 24 per cent sodium chloride was reported by Pyl and Klenk.[16] Rumiantsev[17] stated that the virus survived for at least 26 days in raw hides preserved with sodium chloride. Zhytenko[24] quoted Dronin as having isolated the virus from salted tongues 14 days after slaughter.

MATERIALS AND METHODS

Preparation of Virus.—The virus used was FMDV, Vallee A type, strain 119, isolated in 1932 in Great Britain from an epizootic in cattle. This strain had been carried through 78 bovine serial passages by the British workers.[4] In 1954, the virus was imported to Plum Island and subsequently five additional passages were made. In preparation for this study, the virus was passed twice through cattle, and then inoculated on the tongues of 4 steers to provide sufficient quantities of infected tongue epithelial tissue. The tissue was harvested, pooled, minced with scissors, packed in quantities of about 10 Gm. and stored at —30 C. For use, the tissue was ground in a mortar with 60-mesh alundum. Tryptose phosphate broth of pH 7.4 was added to make a 10 per cent suspension. The material was centrifuged at 880 x g for ten minutes and the supernatant fluid was collected. Penicillin G sodium, 10,000 units, and dihydrostreptomycin, 10 mg., were added for each milliliter of the virus suspension.

Preparation of Tissue Samples.—Many of the tissue samples tested for the presence of virus were prepared in a manner similar to that described for the tongue tissue, and antibiotics in the concentration specified were added to all samples except citrated blood. Sodium citrate was used in the proportion of 20 mg./ml. of blood drawn from the jugular vein. The meat blood samples were composed of small residual blood clots removed from the selected arteries and veins and scrapings from the lining of the vessels. This material was diluted about 1:20 with broth. The bone marrow was removed with a scissors blade from sawed sections about 1 inch long, taken from the vertebral ends of the last four ribs, and diluted about 1:10. The lymph nodes were trimmed of fat and capsule and prepared similar to the other samples except that the ground tissue was placed in a muslin bag in a Pexton Press* and the expressed fluid was diluted 1:10 with broth.

The muscle samples weighed approximately 100 Gm. for the preliminary experiments and 350 Gm. for all others. They were trimmed of excess fat, passed through a meat grinder and then placed in the press. The undiluted expressed tissue fluids constituted the inoculum. The hydrogen ion concentration was determined for all samples, using the Beckman Model G pH meter with glass electrodes.

Dosage of Test Material.—The prepared tissue samples were tested in cattle, using 2.0 ml. distributed on the tongue and from 1.0 to 9.5 ml. given via the intramuscular (i.m.) route. The amount given i.m. depended upon the yield of material from samples. In the preliminary experiments the i.m. route was not used.

Experimental Animals.—Grade Hereford cattle were used—64 steers and 8 heifers as test animals and 7 steers as donors. They averaged 700 lb. and were 18 to 20 months old. They had a fair finish and the carcasses would qualify for market grade, U.S. good. They were housed as experimental pairs in animal isolation rooms, and extreme care was exercised to prevent cross-contamination between

* Lee Engineering Co., Milwaukee, Wis.

rooms. Feeding and watering was accomplished without entering rooms. Chopped alfalfa hay was used. The animals were examined daily for signs and lesions. Those that developed FMD were disposed of after secondary lesions were formed. Those that showed no evidence of infection were held 14 days and then challenged on the tongue, using 2.0 ml. of virus suspension diluted 1:10. Titration challenges were used in only four instances. Blood samples were taken from the animals before inoculation and again on the fourteenth day, so that any doubtful cases could be further studied by serological means.

Preliminary Experiments.—Previous (unpublished) work on Plum Island with strain A-119 had shown that a viremia may be present within 18 hours after inoculation and that secondary lesions appeared after 30 to 34 hours. Accordingly, 2 steers were inoculated on the tongue with 2.0 ml. of virus suspension and 32 hours later they were slaughtered. They had developed typical signs and lesions of FMD. Citrated blood was taken from each animal for titration in 2 test animals. Eight muscle samples were tested on the tongues of 2 steers, taking one sample from each of the quarters of both donor animals. In addition, about 80 lb. of meat was taken from the two carcasses and packed in a wooden barrel, as salted fresh tissue. After storage for 33 days at 4 C., the muscle tissue was tested in 2 steers.

Identification and Distribution of Samples for Main Experiments.—The main experiments were designed so that comparable samples of muscles, blood vessels, and lymph nodes, anatomically identified, would be present in the respective lots of the 24 main experimental groups. Table 1 gives the distribution and donor animal source of the various samples.

The four muscles selected for use (supraspinatus and semitendinosus) were taken from 2 donor animals. Each muscle was divided transversely into four nearly equal parts. The resultant 16 pieces of muscle tissue were distributed so that each of eight experimental groups received one piece from each donor.

The brachial and external iliac arteries and veins from 4 donors were used. An attempt was made to retain blood clots and serum by ligating these vessels and keeping the surrounding tissues with the specimen.

The lymph nodes were taken from 3 of the donors and distributed so that each of eight experimental groups received three anatomically different nodes, using one from each of the donors. The nodes were removed *in situ*, retaining surrounding fat and connective tissues.

Donor Animals.—Five steers, identified by ear tag numbers in table 1, were inoculated on the tongue with 2.0 ml. of a 1:10 dilution of virus suspension. They were also given 2.5 ml. of the material intramuscularly. Thirty hours later, all of the animals had signs and lesions typical of infection with FMD: elevated temperatures (105.2 to 106.8 F.), slight salivation, unruptured vesicles on the tongue and beginning secondary lesions on all four feet of each animal. From 33 to 35 hours after inoculation, the animals were slaughtered and bled out. To avoid contamination with vesicular fluid, the head and feet were removed and discarded from each carcass before skinning and butchering. The carcasses were elevated on a beef tree for skinning and quartering, using standard packinghouse techniques.

Quarters to be used for fresh samples were immediately boned, removing the selected samples as the first operation. Quarters designated for ripening prior to sample collection were held at 20 C. for one hour and then were stored at 4 C. for 72 hours.

Samples Stored Uncured and Cured.—The stored uncured meat was composed of samples of muscles, blood vessels, and lymph nodes taken as fresh tissue and packed without salt in new, paraffin-lined, oak barrels. Three barrels were used, one for each storage period of 16, 30, and 50 days. The samples were identified with wooden labels attached with

TABLE 1—Composition and Source of Samples for 24 Experimental Groups Taken from Donor Steers Infected with Food-and-Mouth Disease Virus

		Tissues from donor animals							
		Muscles		Blood vessels		Lymph nodes			
Treatment groups	Length of storage	Semitendinosus (¼)	Supraspinatus (¼)	External iliac	Brachial	Popliteal	Prefemoral	Internal iliac	Prescapular
Fresh tissues at 20 C.	3 hours	1879-R*	1876-L*	1879-R	1881-L	1879-R	1881-L	.....	1876-L
Ripened at 4 C.	72 hours	1879-L	1876-R	1879-L	1876-R	1879-L	1876-R	1881-R	.....
Samples packed in barrels and held at 1 C.									
Unripened, unsalted	16 days	1879-R	1876-L	1876-L	1879-R	.....	1876-L	1881-L	1879-R
Ripened, salted	16 days	1879-L	1876-R	1881-R	1879-L	.....	1879-L	1876-R	1881-R
Unripened, unsalted	30 days	1879-R	1876-L	1879-R	1876-L	1876-L	.....	1879-R	1881-L
Ripened, salted	30 days	1879-L	1876-R	1876-R	1887-L	1881-R	.....	1879-L	1876-R
Unripened, unsalted	50 days	1879-R	1876-L	1881-L	1876-L	1881-L	1879-R	1876-L	.....
Ripened, salted	50 days	1879-L	1876-R	1887-L	1881-R	1876-R	1881-R	.....	1879-L

* R designates right quarter, L designates left quarter, from which sample was taken. Number indicates ear tag of donor animal.

string and were packed near the middle of each barrel. Additional meat from the infected carcasses was used to fill the barrels. The pieces weighed from 1 to 10 lb. and the total weight of the meat in the barrels averaged about 83 lb. The cured meat was composed of similar samples taken from the ripened quarters. This meat was packed with a salt mixture sprinkled over each successive layer of meat as it was added to the barrels. The salt mixture was applied at the rate of 1 lb. to 25 lb. of meat and was composed of 3 lb. of noniodized, food-grade sodium chloride mixed with 4 oz. of sodium nitrate. In all other respects, the packing of the cured samples was similar to the uncured samples. Immediately after packing and sealing, the barrels of meat were stored in a cooler at 1 C.

In addition to testing the various tissue samples, the bloody tissue fluid which collected at the bottom of the barrels also was tested. On the nineteenth day after slaughter, this fluid was removed from barrels of cured and uncured meat. These barrels previously had been opened and resealed after removal of the meat samples stored for 16 days.

Samples from Ripened Carcasses.—The left forequarter of carcass 1861 and the right forequarter of carcass 1887 were left hanging in the cooler at 4 C. until used in these tests. Rib bone marrow samples were taken from No. 1861 at 14 days postslaughter, and from No. 1887 at 60 and 73 days. Muscle samples were also taken from carcass No. 1887 at 60 and 73 days. The 60-day sample included portions of the supraspinatus and longissimus dorsi. The 73-day samples utilized similar portions of these same muscles but as separate tests of the two muscle areas. From carcass 1887, 60 days postslaughter, the prescapular, axillary, and posterior cervical lymph nodes were used as a test sample. At the same time, blood clots were removed from the axillary and brachial vessels and utilized as another sample.

RESULTS

Virus Titer and Identification.—The epithelial tissue, used as a source of virus for these experiments, had a titer of $10^{7.84}$ bovine i.d.$_{50}$/ml. Tests were made to assure the identity of the virus, using antiserums of FMD types A, O, and C, and vesicular stomatitis types Indiana and New Jersey. These materials were employed in the complement-fixation test in which the 50 per cent endpoint was determined by the method of Brooksby.[3] The antigens were suspensions of the epithelial lesions from the recipients of: (1) fresh muscle, (2) ripened lymph nodes, and (3) rib bone marrow stored 73 days. For each antigen, a strong positive reaction was obtained with FMDV, type A antiserum, and negative reactions with the others. The donor animals in all experiments developed signs and lesions typical of FMD. Likewise, the signs and lesions developed by the test animals that became infected were considered typical for the disease.

Preliminary Experiments.—Citrated blood from the 2 original infected donor animals, taken 32 hours postinoculation, was titrated. The material was used undiluted, and diluted 10^{-3}, 10^{-4}, and 10^{-5}. The blood of 1 animal had a virus titer of 10^{5} and the other a titer of 10^{4} bovine i.d.$_{50}$/ml. The tissue fluid expressed from the eight fresh muscle samples taken from these same 2 donor animals gave positive reactions in all tongue inoculation sites. Thus, it was determined that a viremia was well established within 32 hours postinoculation and that virus was present in muscle tissue taken from various parts of the carcasses at random. The muscle samples had been taken carefully to avoid inclusion or contamination by lymph nodes and large blood vessels. A barrel containing meat from these carcasses, packed fresh with salt mixture added, was tested 33 days after storage at 4 C. Two test animals receiving expressed fluid (pH 5.7) from these meat samples remained normal for an observation period of 14 days.

General Observations.—Quarters from the 5 infected donors and 2 normal steers were used to determine temperature and pH changes during the ripening period. The readings obtained with infected and normal material were similar and, for this reason, both were used to construct figure 1. The quarters were held at room temperature (20 C.) for an hour and then were placed in a cooler at 4 C. The exterior temperature of the meat (not shown on the figure), at a depth of ½ inch, dropped to 10 C. in five to six hours and to 4 C. in 24 hours. The interior temperature, taken at a depth of 5 inches, did not reach 4 C. until nearly 72 hours after slaughter. These temperature variations altered the rate of chemical change at different depths in the meat and thereby affected the pH readings in these areas.

Transverse slices of meat, 1 inch thick and about 6 inches deep, were taken from various areas of the carcasses at the hours specified. The pH electrodes were pressed into the freshly cut surfaces of the muscle tissue and a total of 63 slices were examined. It was noted, with both infected and

normal muscle tissue, that the pH readings were nearly always highest near the superficial edge of each transverse section. As the electrodes were moved inward toward the deep edge, the readings became progressively lower. This was in keeping with the degree of cooling of the various muscle depths. Consequently, four to eight pH readings were taken from each of three areas on each slice: superficial, middle, and deep. The curve labeled superficial represents the highest pH readings obtained in the superficial areas, and the deep curve indicates the lowest readings from the deeper areas. The median curve represents the midpoint of observed pH values and is not the arithmetical mean. The deep and median pH values are more closely related to the temperature curve than are the superficial readings. *Rigor mortis* was detectable about three hours after death, but its peak was not definitely determined. However, the greatest level of acidity was reached in 72 hours. After 72 hours, the pH readings in the various areas became more erratic, but the general trend was upward.

Virus was present in fresh muscle tissue tested about three hours after death. This tissue had been held continuously at 20 C. and had a pH of 5.9, which is more acid than the median shown on the curve at the corresponding time. Virus was not found in the ripened muscle sample tested at 72 hours, and the pH of this sample had reached the 5.5 level, which agreed with the median readings. In the interval between these two tests, the virus was inactivated by the increasing acidity and other chemical changes. The virus population was probably greatly reduced within eight hours, as indicated by the pH curves. But infectious virus may have persisted in the superficial portions of the muscles for nearly 48 hours, since the pH was still 6.0 or higher in some areas.

Hydrogen ion determinations were not made for blood or bone marrow in the hanging carcasses. Lymph nodes that were examined, however, maintained pH readings between 6.4 and 6.9, a favorable range for virus survival.

Meat that was packed fresh in barrels, without salt and without previous chilling, was placed in a cooler at 1 C. for storage. The meat in the center of these barrels remained above the cold room temperature for several days. This undoubtedly allowed a certain amount of ripening to take place within the barrels. This ripening was indicated by the pH values for the meat samples in the barrels that were held for various periods and for the barrel fluids after 19 days of storage (tables 2, 3). In each instance, the values for the ripened and salted samples were slightly higher than those for the unripened and unsalted muscle samples.

The animals were well bled out. Consequently, the residual blood clots and serum that were available for sampling were limited. Thus, pH determinations could not be made until after the blood samples had been suspended in tryptose phosphate broth (pH 7.4). Similarly, the bone marrow samples were suspended in broth prior to taking pH readings, because of the limited amounts of extractable fluid. However, these tissues were considered to be well buffered and the pH change brought about by the broth may not have been great. Comparative readings obtained from lymph node fluid before and after dilution with broth showed very little change in the pH values (<0.2).

Comparative Tests.—The donor animal source of the anatomical parts used and the distribution of the comparable samples in the 24 main experimental groups are given (table 1), as well as the results of the tests (table 2).

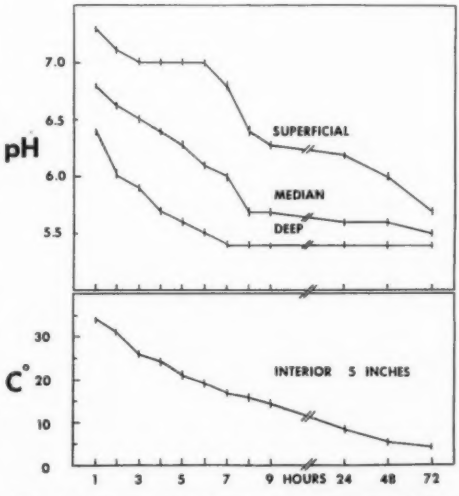

Fig. 1—The pH and interior temperature changes in muscles of steer carcasses during ripening at 4 C.

TABLE 2—Survival Periods of Foot-and-Mouth Disease Virus in Muscle, Blood, and Lymph Nodes Held as Cured and Uncured Meat

Treatment of samples	Length of storage	Tissue samples: Muscle pH	Muscle No. positive	Blood *pH	Blood No. positive	Lymph node pH	Lymph node No. positive
Fresh at 20 C.	3 hours	5.9	2/2**	7.3	2/2	6.9	2/2
Ripened at 4 C.	72 hours	5.5	0/2	7.3	2/2	6.6	2/2
Packed in barrels and held at 1 C.:							
Unripened, unsalted	16 days	5.6	0/2	6.9	0/2	6.2	2/2
Ripened, salted	16 days	5.7	0/2	6.8	0/2	6.5	0/2
Unripened, unsalted	30 days	5.5	0/2	6.6	0/2	6.4	2/2
Ripened, salted	30 days	5.8	0/2	7.1	0/2	6.4	2/2
Unripened, unsalted	50 days	5.5	0/2	7.5	0/2	6.1	2/2
Ripened, salted	50 days	5.7	0/2	6.6	0/2	6.4	2/2

* The pH of blood samples influenced by addition of broth (pH 7.4). ** Numerator = number of steers positive: denominator = number of steers used.

The animals inoculated with the various muscle tissue samples all remained normal for the observation period of 14 days, except those that received fresh muscle inoculum. All animals inoculated with fresh tissue samples from muscle, blood, or lymph nodes developed FMD. The fresh and ripened samples of blood gave positive findings, but samples stored in barrels, with and without the salt mixture, gave negative results.

The animals inoculated with lymph node samples all developed the disease, regardless of the method of sample treatment and the length of storage period. The exceptions were 2 steers that had received the sample that had been ripened and salted and stored for 16 days. Difficulty was experienced in expressing fluid from the lymph node samples that had cured 16 days, and the material was processed considerably longer than other samples. Failure to demonstrate the virus in this test may have been caused by the altered technique, by unequal virus distribution in donors, or by some undetected accident. The fact that the virus was present in cured lymph nodes held for 30 and 50 days indicates that it also should have been detected in the 16-day sample.

TABLE 3—Survival of Foot-and-Mouth Disease Virus in Stored Meat Fluids and Ripened Steer Carcasses

Description and treatment of sample	No. of days stored	pH of sample*	No. positive
1. Tissue fluids drained from meat in barrels stored at 1 C.:			
A. Unripened, unsalted	19	5.4	0/1**
B. Ripened, salted	19	5.6	0/1
Tissues from hanging carcasses ripened and stored at 4 C.:			
2. A. Rib bone marrow	14	7.6	2/2
B. Rib bone marrow	60	7.1	2/2
C. Rib bone marrow	73	7.5	2/2
3. Muscle tissue:			
A. Supraspinatus and longissimus dorsi	60	6.0	1/2
B. Supraspinatus only	73	6.6	0/2
C. Longissimus dorsi only	73	6.4	0/2
4. A. Lymph nodes	60	6.7	2/2
B. Blood	60	7.1	2/2

* The pH of bone marrow and blood samples influenced by broth suspending medium. ** Numerator = number of steers positive; denominator = number of steers used.

No antibodies were detected in the serum of these animals taken 14 days postinoculation with neutralization tests used in suckling mice. Similar tests with serum obtained from other test animals that had failed to develop FMD after inoculation with muscle and blood samples, also gave negative neutralization results. A representative group (about 1/3) of these 14-day postinoculation serum samples was tested in this manner.

Barrel Fluids and Samples from Ripened Carcasses.—The results of these experiments are given (table 3). The virus was not found in the bloody fluid that had drained from the meat and collected in the bottom third of the barrels. Tests were made on both the salted and unsalted samples after 19 days of storage. In samples taken from two forequarters of donor animal carcasses that had been continuously stored at 4 C., virus was detected in the rib bone marrow at 14, 60, and 73 days. Likewise, the virus was present in lymph nodes and in large blood clots from the axillary region when tested at 60 days. A composite muscle sample taken from the same carcass that furnished the lymph node, blood, and marrow samples noted previously also had virus present when tested at 60 days. Only

1 of the 2 steers became infected, possibly indicating a low level of virus concentration in the sample. However, this may have been due to the accidental inclusion of small lymph nodes, protected blood clots, or fragments of bone and marrow from the sawed vertebrae or to the survival of virus near the superficial surface of the meat. Muscle samples were retested after 73 days' storage. The samples used were adjacent areas of the same two muscles, the longissimus dorsi and supraspinatus, but were tested separately. In these trials, virus was not detected. The longissimus dorsi is in a position to be contaminated by vertebral bone and marrow fragments after slaughter when the carcass is sawed or cut in half.

Discussion

The literature provided sufficient data to formulate an opinion as to the probability of virus being present in cured meat commercially prepared in areas where FMD is enzootic; but actual experimentation had not been done in which commercial practices were closely followed. It was known from the work of the British [6] and from that of Pyl and Klenk [16] that various salt mixtures used for curing meat did not inactivate the virus. Henderson and Brooksby [9] had been unable to recover virus from muscle tissue stored for three and eight weeks at 4 C., but the virus was present in defibrinated blood samples stored at the same temperature for six weeks. Various studies emphasized the importance of lymph nodes and bone marrow as tissues offering conditions conducive to virus survival.

It was believed that maximum conditions should prevail in order to give the natural virus-inactivating process an adequately severe test. For this reason, the infected donor animals were purposely slaughtered at a time considered to be near the peak of virus production and viremia. This assured an adequate virus distribution throughout the body and tended to make the samples more nearly comparable in initial virus content. Negative test results would have little value if the virus was not well distributed. Virus was recovered from all fresh tissues tested, which proved that a good distribution had been achieved. Titrations of the samples were not made because a maximum dosage of available material was given to each test animal; detecting the virus, if present, was considered to be more important than determining the quantity present.

The infected donor animals did not show lameness from the secondary lesions, which were just developing at time of slaughter. Salivation was slight and the outward signs of illness were such that a market animal similarly involved might escape notice in a casual antemortem inspection. Veterinary inspectors generally agree that a clinical diagnosis of suspected foot-and-mouth disease is difficult to make in animals seen only briefly. Such a diagnosis, of course, would require laboratory confirmation. Animals in the stage of infection described, as well as in other stages, could be used inadvertently as a source of meat.

The muscle tissue of boned meat is probably rendered free of active virus in about three days when stored at temperatures above the freezing point of water. Apparently, the virus in small residual blood clots and on vessel walls is also inactivated in boned meat taken from well-bled animals. The addition of a salt mixture to meat causes more fluids to be withdrawn from the tissues than in unsalted meat. This withdrawal may slightly hasten the inactivation process; there is no evidence that it retards inactivation. Fresh-boned meat packed in a barrel and the deep portions of muscles on a carcass give up heat at a slow rate. This allows the chemical changes to take place more rapidly in these insulated areas than in the superficial portions. The pH readings demonstrate this fact, and indicate that conditions are favorable for virus survival near the superficial surface of carcass muscles for nearly 48 hours when the carcass has been rapidly chilled. Thus, to hasten the inactivation process, the muscle tissue should be cooled as slowly as possible, still retaining the keeping quality of the meat. However, little would be gained in virus inactivation by continuing the ripening period beyond 72 hours.

Nature has provided an effective mechanism for inactivation of the virus in muscle *per se*. Unfortunately, the problem does not end at this point. Boned meat, as customarily prepared, also contains lymph nodes and large blood vessels. Occasionally, large blood clots are present and also fragments of bone. Muscles taken from the vertebrae may be particularly contaminated with bone, because they are near the point where the carcass is split. Lymph nodes,

large blood clots, and bone marrow are well buffered. They seem to provide physical and chemical barriers that prevent lactic acid and other substances from penetrating and inactivating the virus.

From a practical standpoint, it would be nearly impossible for any abattoir to provide cured meat products free of such tissues, which are conducive to virus survival. It would also be impossible to guarantee in an enzootic area that infected animals would never be used for meat. Therefore, the virus may survive and be demonstrable in commercially boned cured or uncured meat, if the meat were obtained from an area where foot-and-mouth disease is present.

SUMMARY

Tests were conducted to study the survival of foot-and-mouth disease virus in cured and uncured meat. Meat samples were tested in 37 experimental groups of cattle or a total of 72 test animals. Comparable and anatomically identified samples were taken from the carcasses of infected donor cattle. The donor cattle were slaughtered 33 to 35 hours after inoculation with foot-and-mouth disease virus, type A, strain 119, at a time when viremia was established and secondary lesions were beginning.

The meat samples were tested fresh, after ripening (stored 72 hours at 4 C.), and after storage in wooden barrels at 1 C. for 16, 30, and 50 days. Each of the latter included barrels of cured meat (ripened and salted) and barrels of unripened, unsalted meat. Samples of muscles, blood, and lymph nodes were tested in each instance. Infective virus was demonstrated in all fresh samples, in ripened blood and lymph node samples, in unripened, unsalted lymph node samples stored 16, 30, and 50 days, and in cured lymph nodes held for 30 and 50 days.

Carcasses tested after storage at 4 C. contained demonstrable virus in rib bone marrow at 14, 60, and 73 days, and in lymph nodes, blood, and muscle samples at 60 days. Muscle samples taken after 73 days' storage were negative.

The chemical changes that take place during the ripening of meat inactivate the virus in muscle tissue but, apparently, they do not appreciably affect the virus in lymph nodes, large blood clots, or bone marrow. In transverse sections of muscle tissue taken from rapidly chilled carcasses, the pH readings show a general increase in acidity from the superficial toward the deep areas. Conditions are more favorable for virus survival in the superficial portions for nearly 48 hours.

It was concluded that meat derived from animals infected with foot-and-mouth disease was not rendered free of the virus by the usual commercial procedures of ripening, boning, salting, and storage. The study showed that the virus could survive in certain tissues customarily included in such meat.

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Morphologic and Histochemical Studies of Skeletal Lesions in Rats Fed Sweet Pea (*Lathyrus odoratus*) Seeds

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DESPITE THE WELL-KNOWN and striking effects of lathyrism on the skeleton, relatively few histochemical reports are available concerning the possible relationship of experimental lathyrism to the rapidly growing regions of long bones. The effects of *Lathyrus odoratus* diets on the epiphysial plates of rats have been variously described as a proliferation of cartilage cells with an irregular zone of calcified cartilage and retardation of endochondral ossification [18]; a loss of orientation with clumps of chondrocytes scattered throughout a voluminous abnormal matrix showing degeneration of the ground substance as exemplified by fragmentation of the matrix [14]; and a loss of cohesion of the cartilage matrix and loosening and detachment of the tendinous and ligamentous insertions.[16] The toxic factor in lathyrism was first extracted in impure form [21]; subsequently it has been possible to demonstrate that *beta* (*gamma*-L-glutamyl) aminopropionitrile is present in the seeds of *L. odoratus* and duplicates the effects of this plant on the skeleton.[2, 3, 19]

This study was undertaken to investigate the morphologic and histochemical effects of *beta*-aminopropionitrile, as present in sweet pea seeds, on the histologic evolution of skeletal deformities. Often in the study of lathyrism and related disorders more has been expected of and claimed for morphologic and histochemical methods than they could be reasonably expected to give; the objectives of this investigation are within the scope of the methods used.

This study also attempted to elucidate the mode of action and the metabolic site at which the sweet pea toxin acts. A detailed histochemical investigation of the alterations in lathyrism indicates that the toxin produces a specific change in mesodermal tissues,[5-8] particularly the growth cartilage of long bones. The seed is therefore acceptable for use in the investigation of the metabolism of hyaline growth cartilage. An insight into the diseases influencing hyaline growth cartilage presupposes an understanding of the metabolism of other mesodermal tissues. With this background in mind, the histochemical study of the effect of *L. odoratus* feeding on the epiphysial growth plate was undertaken.

MATERIALS AND METHODS

The albino rat was used because it has been used extensively in stress and hormone studies related to lathyrism; also, the rat is extremely susceptible to lathyrism and can easily be fed different diets.

Forty-eight male albino rats of the Holtzmann-Sprague-Dawley strain, weighing 45 to 50 Gm., were allotted to one group at weaning and fed diets containing 50 per cent *L. odoratus* seeds (principals). An additional 48 rats were fed similar amounts of two control rations and served as normal litter mate controls. Each principal was paired with a normal control of the same weight. Control animals were killed at the same time of day as the principals. The rats were housed separately in wire-bottomed cages in an isolated room maintained at the usual laboratory temperature (70–80 F.).

Experimental Diet.—The experimental diet contained *L. odoratus* meal, 50 per cent; untreated casein, 10 per cent; cornstarch, 25 per cent; sucrose, 5 per cent; brewer's yeast powder, 4 per cent; salt mixture,[10] 4 per cent; and cod liver oil, 2 per cent. The whole dried peas were ground to a fineness that permitted all the pea meal to pass through a 24-mesh sieve. The dry ingredients of the diet were mixed and stored; cod liver oil was added to portions of the mixture as needed.

One control diet contained the same ingredients as the experimental diet, but a 50 per cent dried and ground commercial edible pea meal replaced the *L. odoratus* meal; the other control diet was a milled rat diet.*

The principals and their controls were euthanatized after one, two, or three weeks to determine the progressive effects of a 50 per cent *L. odoratus* diet. Pilot studies had determined the survival time for rats on this diet; three weeks of feeding was sufficiently in advance of the expected time of death to avoid drastic terminal degenerative changes, so that histochemical data were uncom-

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* Rockland rat diet, A. E. Staley Manufacturing Co., Chicago, Ill.

plicated by these factors. All rats had developed lathyrism but were still active.

Necropsies were performed immediately. The carcasses were skinned and eviscerated; long bones were removed, radiographed individually, and fixed in Zenker's formalin solution for one day or in Bouin's solution for two days to preserve histologic details.

Histologic Preparation and Analysis.—After fixation, gross tissue sections were decalcified by placing the long bones in cold ethylenediaminetetracetic acid (EDTA) at a pH of 7.0 for six days. To overcome any processing inadequacies and artifacts the paraffin-celloidin, double-embedding method was used, slowing down the progression to absolute alcohol and prolonging the treatment in celloidin-methyl benzoate to three days. The final paraffin treatment was limited to four to six hours.

Consecutive, 6- to 8-μ histologic sections of long bones were cut longitudinally and stained with Harris' hematoxylin and eosin Y, Mallory's phosphotungstic acid-hematoxylin, periodic acid-Schiff (PAS), Masson's trichrome, toluidine blue, crystal violet, Wilder's reticulin, or van Gieson's stains. After mounting in Canada balsam, the cover slips were weighted to obtain flat sections.

RESULTS

The lesions of lathyrism were primarily lesions of the growing portions of long bones and were best developed in the epiphysial cartilage and other areas where formation and deposition were taking place.

NORMAL EPIPHYSIAL CARTILAGE OF LONG BONES

The structure of the epiphysial cartilage in the long bones of the rat may be divided into the following zones: (1) layer of germinal cells; (2) layer of proliferation; (3) layer of chondrocyte growth; (4) layer of intracellular edema; (5) layer of calcification; and (6) layer of destruction. Subjacent to the growth cartilage is the primary spongiosa of the metaphysis.

The chrondrocytes are regularly arranged in columns (fig. 7), one chondroycte in each lacuna, with relatively scanty matrix between the lacunae. Within the lacunae, the chondrocytes are embedded in ground substance which is metachromatic. This material cannot be demonstrated by PAS. The matrix of the cartilage shows a rich network of fine argyrophil fibrils, which correspond to the collagen structure as revealed by the electron microscope. The epiphysial cartilage grows mainly by interstitial growth but also by minor appositional growth. The cartilage matrix of normal rats has a uniformly homogeneous metachromatic staining (table 1).

EPIPHYSIAL CARTILAGE OF PRINCIPALS

Upon histologic study of sections stained with hematoxylin and eosin Y, a typical irregular proliferation of the epiphysial cartilage plates was the most pronounced finding (fig. 1).

There was an increase in the size of the chondrocytes as well as a great increase in their number (fig. 1). Some cells were undergoing mitosis. The chondrocytes were no longer regularly arranged in orderly rows, but they appeared in territorial clusters or groups. The groups were separated from each other by wide septa of deeply eosinophilic cartilage (fig. 2). There was an irregular proliferation of cartilage cells

TABLE 1—Staining Reactions of the Alterations in the Matrix of Epiphysial Cartilage During Experimental Lathyrism

Histochemical stain	Lathyric cartilage matrix	Normal cartilage matrix (principals)
Harris hematoxylin and eosin Y	Many linear areas and streaks of eosin-stained matrix	Uniformly homogeneous eosin staining matrix
Periodic acid-Schiff	Many linear areas and streaks of strongly PAS-positive reddish purple matrix	Uniformly weak PAS positive, pale pink intercellular matrix
Masson's trichrome stain	Many streaks and areas of brilliant blue collagenous staining matrix	Uniformly homogeneous light blue stain for cartilage matrix
Toluidine blue	Many streaks and areas of negative metachromasia	Matrix homogeneous metachromasia
Crystal violet	Negative metachromasia	Uniformly metachromatic
Mallory's phosphotungstic acid-hematoxylin	Many areas and streaks of dense brownish red staining matrix	Uniformly homogeneous light brownish red matrix
Wilder's reticulin	Many streaks and areas of a dark reddish brown network of fine fibrils in matrix	Uniformly homogeneous light brown staining matrix
Van Gieson's stain	Many streaks and areas of dark red staining matrix	Uniformly homogeneous light red matrix

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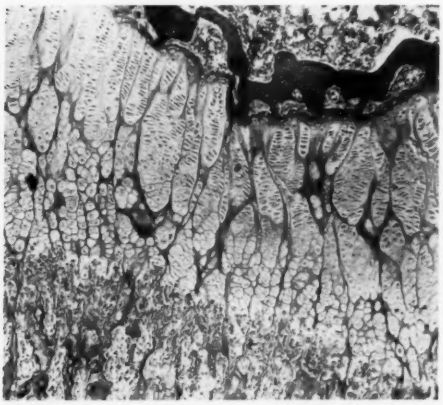

Fig. 1—The epiphysial plate from the tibia of a male rat fed a 50 per cent sweet pea diet for three weeks. Notice the increase in width of the epiphysial plate, the marked increase of the zone of chondrocyte growth, a disorganization of all zones, and the linear areas in the matrix staining densely with eosin. H & E stain; orig. mag. x 35.

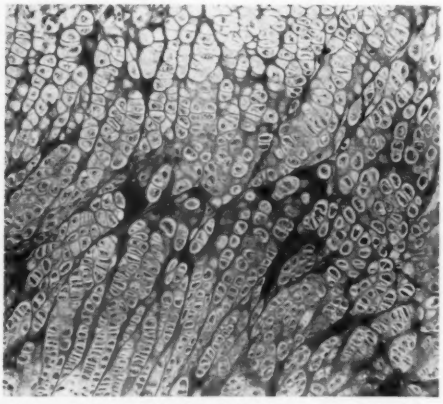

Fig. 2—The epiphysial plate from the tibia of a male rate fed a 50 per cent sweet pea diet for three weeks. Notice the many linear areas and streaks of matrix separating clusters of chondrocytes. The streaks stained purple red with eosin. H & E stain; orig. mag. x 50.

with a chondroma-like appearance. Therefore, a premature cessation of growth in length resulted. The cartilage matrix was granular, no longer appearing cohesive, and became somewhat fibrillated in areas deep in the growth cartilage (fig. 3).

Throughout the zone of chondrocyte growth, areas of the cartilage matrix appeared to have disintegrated (fig. 3). The degeneration was particularly prominent in the plane between the zone of proliferating cells and the zone of calcification.

Capillaries invaded the lateral and middle zones of the epiphysial cartilaginous plate, and eventually marrow cavities (fig. 4, 7) developed in focal areas of the hyaline cartilage. The columnar arrangement of chondrocytes in the zone of calcification was completely lost. Calcification of the deep zone of the cartilage adjacent to the metaphysis did not appear to be greatly disturbed. Endochondral bone formation was occurring at the metaphysis (fig. 5). No osteoid seams were seen in the metaphysis. The bone marrow was normal.

Some of the primary bone trabecule had disappeared in the metaphysis adjacent to the zone of destruction of the cartilage.

The articular cartilage showed a few areas of fibrillar degeneration (fig. 6). The cartilage matrix stained red with eosin in areas deep in the joint cartilage. There was formation of some deep nests of cartilage cells. The subchondral plate was disrupted and irregular. The underlying subchondral bone had disappeared. The few remaining trabeculae of bone consisted of an irregular core of calcified cartilage covered by calcified lathyric bone. Osteoid borders were consistently absent.

HISTOCHEMICAL CHANGES IN THE EPIPHYSIS

The matrix of hyaline cartilage in PAS-stained preparations showed linear areas and streaks which were strongly PAS-positive. This reddish purple accumulation of intercellular material showed a fibrillar-type pattern and no longer exhibited the pale pink (PAS-negative) and homogeneous material as in normal hyaline cartilage matrix.

The chemical nature of this material was, however, unknown. Isolated acid mucopolysaccharide does not react, or reacts very slowly with periodic acid. Thus, the material was probably not isolated acid mucopolysaccharide.

The same streaks and linear areas in the matrix that were strongly PAS-positive were negative to metachromatic staining with toluidine blue and crystal violet.

Masson's strong blue positive material steadily increased in amount in the interlacunar cartilage matrix. The matrix showed loss of its homogeneous appearance with fibrillation. The accumulation of dark blue material in the matrix was consistent

with either increased deposition of normal collagen or the deposition of an abnormal type of soluble collagen.

The accumulated interlacunar material showed a complete breakdown of the matrix with irregular masses of fine fibrils between cartilage clumps in preparations stained with Mallory's phosphotungstic acid-hematoxylin. The many linear areas of brownish-red intercellular material were consistent with increased collagen deposition or accumulation of tropocollagen in the cartilage matrix.

With Wilder's reticulin stain, the fibrillar-like pattern of the matrix was readily visible. It consisted of a rich network of fine fibers and fibrils between the clumps of cartilage cells. The intercellular material stained with a reddish-brown tone corresponding to a reticulin structure.

The interlacunar matrix of the epiphysial cartilage showed the accumulation of many areas of material strongly positive to van Gieson's stain. Fibrillation of the matrix was also evident with van Gieson's stain.

EXPERIMENTAL NASAL CARTILAGE

The nasal cartilage initially showed intracellular edema of the chondrocytes and a reduction of intercellular substance.

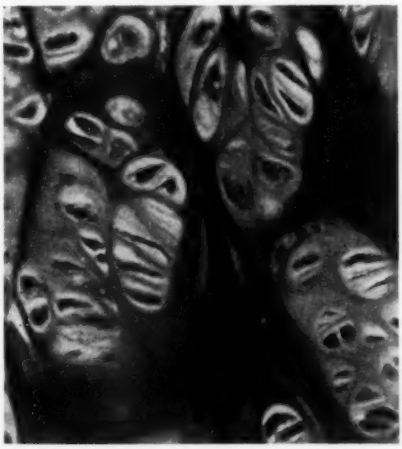

Fig. 3—The epiphysial plate from the tibia of a male rat fed a 50 per cent sweet pea diet for three weeks. Notice the many linear areas and wide streaks of dense matrix which stained deep brownish red. The septa of cartilage matrix are fibrillated. Chondrocytes are arranged in territorial groupings. Mallory's phosphotungstic acid-hematoxylin stain; orig. mag. x 300.

Fig. 4—The upper epiphysial growth plate from the tibia of a male rat fed a 50 per cent sweet pea diet for three weeks. Notice the widened septa of cartilage matrix which stained dark red with eosin. The matrix had degenerated and areas have been invaded by capillaries or perforated by marrow channels, or both. The cartilage matrix is fibrillated. H & E stain; orig. mag. x 375.

The degeneration of the cartilage began within the central portion. In a very short time it was characterized by enlargement of cells, due to the intake of great amounts of fluid which appeared as vacuoles in the cytoplasm and in the nucleus. The edema increased until the cytoplasm eventually consisted of a faintly staining network and the chromatin was displaced to the periphery so that the nucleus became an empty zone. The swelling of the cells led to a stretching of the intercellular substance. The intercellular substance became transformed (fig. 8) into a network staining an uneven purple red with eosin, and appeared to be degenerating (in normal nasal cartilage, the matrix stained a uniform and homogeneous light red with eosin). Although the chondrocytes had undergone lysis, lacunae were still visible and cartilage remained coherent and cohesive (fig. 8).

DISCUSSION AND CONCLUSIONS

It is interesting that in this investigation the animals given *L. odoratus* showed PAS-positive staining of the epiphysial cartilage, characterized by multiple linear areas and streaks of strongly PAS-positive matrix, while the epiphysial plate of normal animals is homogeneous and uniformly PAS-negative. In contrast to the strongly PAS-positive matrix, these same areas showed

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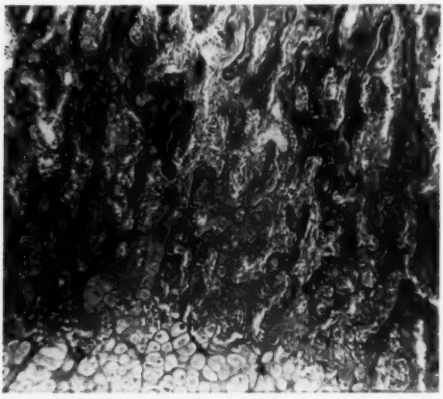

Fig. 5—The metaphysis adjacent to the upper epiphysial plate from the tibia of a male rate fed a 50 per cent sweet pea diet for three weeks. Notice the endochondral bone formation. The trabeculae of the metaphysis consisted of dark cores of calcified cartilage matrix which was never destroyed. This was covered with lathyritic bone showing an absence of osteoid borders. H & E stain; orig. mag. x 35.

negative metachromasis, while normal cartilage is homogeneously metachromatic. Histochemical studies of the ground substance in hyaline cartilage shows that these two stains bear an inverse relationship to one another.[20]

Inasmuch as the accumulation of intercellular material is not metachromatic and is strongly PAS-positive, it is probably not acid mucopolysaccharide.

The degree of color developed by the PAS reaction in epiphysial cartilage is dependent on the amount of reactive glycol structure present in the cartilage matrix. After the use of the ordinary aqueous fixatives, only substances of high molecular weight remain in the matrix in sufficient quantity to give a positive result. The strongly positive PAS reaction signifies the presence of polysaccharide from the 1:2-glycol groups present in collagen. Although normally these reactive groups are not free to react, in lathyrism they are set free from physicochemical forces.

However, on this basis alone we are reluctant to give a definite interpretation to these two histochemical reactions. The two reactions still await a positive histochemical correlation. There is, however, a difference in the PAS staining of young and old cartilage. Loewi [13] interpreted the loss of metachromasia in old cartilage as being due to depolymerization with aging. It is possible that, in the lathyric rat, premature aging of epiphysial cartilage occurs with areas of strongly positive PAS material corresponding to depolymerization of the matrix.

The destruction of the nasal cartilage by the *L. odoratus* toxin is in all probability also a depolymerization of the intercellular substance. The change in staining reaction to a strong eosin-stained intercellular substance may be due to a change in the organic component. The severity of the alterations in the nasal cartilage increased with prolonged experimental feeding.

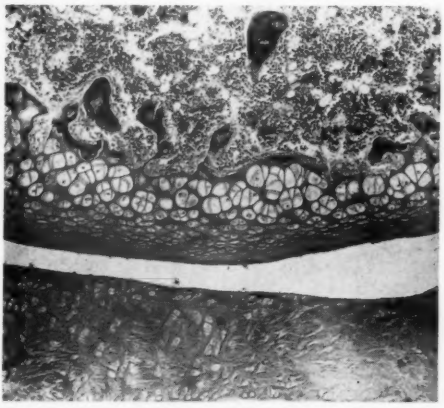

Fig. 6—The articular cartilage of the tibia. The cartilage matrix stained red with eosin, with the formation of nests of cartilage cells occurring in the deeper layers adjacent to the epiphysial bone. The subchondral line was irregular and very few trabeculae of subchondral bone were present along this border. The few remaining trabeculae consisted of dark central zones of calcified cartilage matrix surrounded by bone. H & E stain; orig. mag. x 35.

However, the utilization of other histochemical techniques may be of considerable value in obtaining information on the pathogenesis of lathyrism. By site and staining reaction, the intercellular material appearing strongly PAS-positive is also positive to Masson's trichrome stain, Mallory's phosphotungstic acid-hematoxylin stain, van Gieson's stain, and Wilder's reticulin stain. All of these corroborate the material as collagen, soluble collagen, or tropocollagen showing an absence of metachromasia. With an accumulation of tropocollagen or soluble collagen, the general breakdown of the cartilage matrix takes place.

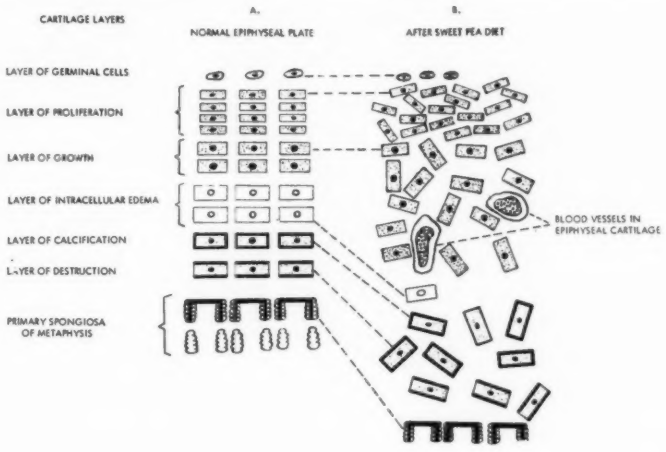

Fig. 7—Diagrammatic representation of the epiphysial alterations occurring in male rats fed a 50 per cent sweet pea diet for three weeks. (A) the normal epiphysial cartilage showing a division into various zones; (B) the lathyritic cartilage showing the severe disorganization, increase in width, invasion of plate by blood vessels, and irregular calcification.

In order to have formation of normal collagen fibers in lathyrism, the constituent amino acids must be in optimal concentrations and a proper linkage must be produced to form the basic collagen molecule, tropocollagen.[9] These molecules then aggregate to form mature collagen fibers. In lathyrism, these two factors are not satisfied. Perhaps the transformation of tropocollagen into mature collagen molecules is disturbed and this produces the basic histologic and histochemical defect in lathyrism.

The collagen content of epiphysial cartilage is not reduced from normal during lathyrism. The collagen content may simply be in the form of the tropocollagen molecule of soluble collagen due to a failure to form fibers. However, an increase in the solubility of components of cartilage in 1 M sodium chloride has been reported by Levene.[12] The action of the physiochemical factors described by Gross[9] on the tropocollagen molecule to form the fibers of collagen is somehow inhibited in lathyrism. The method of inhibition is unknown at the present time.

As a result of the *L. odoratus* feeding, the hyaline cartilage of the epiphysial plate does not calcify properly, whereas proliferation of cartilage progresses for an extended period. The uncalcified cartilage is resistant to resorption, preventing replacement by bone at the metaphysial border. As a consequence of the progressing proliferation and the lack of resorption, the epiphysial plate increases to several times its physiologic width. The normal reconstruction and modelling resorption of the growing bone is, therefore, disturbed. As the changes progress in the growth cartilage, there is a cessation of maturation in the layer of chondrocyte growth. This results in a severely widened and irregular epiphysial plate. The cartilaginous proliferations, however, are noninflammatory in nature. As a result of this influence, there is an interference with endochondral ossification. The zone of developing trabeculae of the metaphysial end is altered

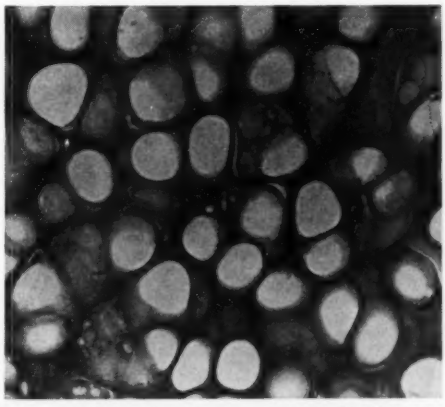

Fig. 8—A frontal section through the hyaline cartilage of the nasal septum of a male rat fed a 50 per cent sweet pea diet for three weeks. Notice that by three weeks the chondrocytes have all died. These cells have undergone lysis, though their lacunae are still visible. The cartilage remained coherent and hyaline. The matrix of the hyaline cartilage showed a dark basophilia. H & E stain; x 300.

and consists of calcified cores of cartilage covered by irregularly calcified lathyric bone. Blood vessels can be observed entering the cartilaginous epiphysis by way of the perichondrium. These small capillary bushes occur in canals invading areas of degenerated cartilage. This inclusion of blood vessels in the epiphysial cartilage is probably a reflection of the eccentric growth occurring in growth centers during lathyrism.

The epiphysial plate lesions of lathyrism are more severe in the more actively growing epiphysial plates, such as the lower end of the femur and the upper end of the tibia. Therefore, one may conclude that there is a selective localization of the effect of the sweet pea toxin to growing cartilage. This appears quite possible, since cartilage is extremely susceptible to any general disturbance in nutrition. Because so little is known of the metabolic activities characterizing epiphysial cartilage, it is extremely difficult to fully understand the derangements in the growth cartilage during experimental lathyrism.

In regard to a correlation of the pathologic alterations with the duration of experimental *L. odoratus* feeding, one readily observes the following: After one week of the experimental diet, the epiphysial plate is slightly irregular with minimal enhancement of interstitial growth. At the termination of two weeks, there is great irregularity of the growth plate with an exaggeration of interstitial growth of chondrocytes. After three weeks of *L. odoratus* feeding, the growth plate is tremendously increased in width, due to enhancement of both interstitial and appositional cartilaginous growth. There is an increase in cartilage matrix which appears fibrillated and separates territorial groups of chondrocytes. The widened septa of cartilage matrix are perforated by blood vessels and bone marrow spaces.

Thus, the action of *beta*-aminopropionitrile, present in the *L. odoratus* pea, is cumulative in nature. The longer the period of experimental feeding the more severe the histopathologic alterations in the epiphysial plate.[1,4,17] No regression of the cartilaginous lesions occurred at any time during this investigation.

One can readily surmise from the observations reported that there is a definite perversion in the epiphysial growth cartilage caused by the feeding of *L. odoratus* to albino rats.

The knowledge gained from the results of this investigation is not sufficient to give a definitive chemical structure for the histologic or histochemical alterations found in lathyrism.

SUMMARY

A histologic and a histochemical study of the epiphysial cartilage deformities produced by feeding *Lathyrus odoratus* peas is presented. The uniform periodic acid-Schiff (PAS) reaction was strongly positive in linear areas and streaks throughout the hyaline cartilage matrix. These same septa were negative to metachromatic staining with toluidine blue and crystal violet. The streaks of cartilage matrix were, however, strongly positive to Masson's trichrome stain, Mallory's phosphotungstic acid-hematoxylin stain, Wilder's reticulin stain, and van Gieson's stain. This was consistent with the deposition of tropocollagen or soluble collagen in the cartilage matrix with a general breakdown of supporting structures.

The epiphysial growth cartilage was widened due to proliferation of the layer of chondocyte growth. The localization of the lesions must be significant, as is the constant development of a strongly positive PAS matrix showing negative metachromasia with strong reactions for collagen. This combination of site and histochemical staining reactions identifies the material in epiphysial cartilage matrix as tropocollagen or soluble collagen, and it is concluded that there is an important connection between the lathyrus factor and collagen.

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XUM

Cerebrospinal Fluid Electrolytes in the Dog

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THIS REPORT is the result of an investigation to determine normal electrolyte values for canine cerebrospinal fluid as control data for a study of the neurotrophic effects of peripheral nerve irritation. A review of the literature revealed that virtually no information had been published on cerebrospinal fluid electrolytes in the dog.

MATERIALS AND METHODS

All animals were processed for experimental use by immunization for canine distemper, canine infectious hepatitis, and rabies, during a quarantine period. Internal and external parasites were eliminated to the maximum extent possible. The dogs were fed a ration of cooked horsemeat and a commercial dog food.*

A total of 129 samples of cerebrospinal fluid was taken from 83 dogs of mixed breed and sex. The dogs were 4 to 8 years old. Aspiration of cerebrospinal fluid was performed on anesthetized dogs by puncture of the cisterna magnum with a 20-gauge, 1½-inch disposable needle. Aseptic technique was used in collection, and repeat sampling was done at intervals of two weeks or longer. Five milliliters of cerebrospinal fluid was aspirated into a 10-ml. syringe. Samples grossly contaminated with blood were discarded. Other samples were centrifuged to check for the presence of erythrocytes.

PROCEDURE AND RESULTS

Sodium and potassium concentrations were determined on a Beckman DU flame spectrophotometer equipped with constant voltage power supply, spectral energy recording adapter, photomultiplier, and burning a hydrogen-oxygen flame. Test specimens were run against 1 per cent trichloroacetic acid working dilutions of a stock serum analysis standard containing sodium, 144.0 mEq/1; potassium, 5.0 mEq/1; calcium, 5.0 mEq/1; magnesium, 2.0 mEq/1; and phosphate, 2.2 mEq/1. Potassium was measured in a 1:10 dilution; a 1:200 dilution was best suited for sodium readings. All test specimens contained 1 per cent trichloroacetic acid.

Chloride determinations were carried out on the Cotlove [1] Chloridometer.* One-tenth milliliter of serum was added to 4 ml. of nitric-acetic reagent, 4 drops of gelatin reagent was added, and the mixture was titrated amperometrically. Silver ions from the generator electrodes combine with chloride until all available chloride is precipitated. Increasing free silver ions in the solution results in an increased current and the preset meter-relay stops the timer. Silver ions are produced at a constant rate and, therefore, the chloride precipitated varies directly with the elapsed time.

Calcium was determined on the Autoanalyzer** by a modification of a method outlined by Moser.[2] The spinal fluid was mixed with a buffered hyamine reagent at pH 4.0 and dialyzed into an aqueous recipient stream. Ammonium purpurate was added to the dialysate and a colored complex was formed between calcium and the indicator. The color was measured at 505 mμ in a 6-mm. light path. The sodium chloride concentration recommended for the standards used in serum calcium determinations, 0.5 M, was reduced to 0.05 M for spinal fluid. This concentration led to re-

TABLE 1—Cerebrospinal Fluid Electrolyte Values for Dogs

Variable	Mean	Standard deviation	95% confidence limits
Sodium (mEq/1)	156.3	7.17	142.0–170.6
Potassium (mEq/1)	3.1	0.17	2.8–3.4
Calcium (mg. %)	6.2	1.22	3.8–8.6
Chloride (mEq/1)			
Phosphate (mg. %)	1.1	0.25	0.6–1.6

Values represent the results of analyses of 129 samples from 83 dogs.

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The opinions expressed herein are those of the authors and are not to be construed as representing official USAF policy.

* Purina Dog Chow, manufactured by Ralston Purina Co., St. Louis, Mo.

* Laboratory Glass and Instruments Corp., New York, N.Y.

** Technicon Instruments Corp., Chauncey, N.Y.

sults in primary studies that more nearly agreed with existing manual methods.

Inorganic phosphate was determined on the Autoanalyzer** by a modification of the method of Fiske and Subbarow.[3] The procedure consists essentially of mixing the sample with NaCl solution, dialysis, addition of acidic ammonium molybdate, and reduction with 1-amino-2-naphthol-4-sulfonic acid. After circulation through a heating bath, the color was read at 660 mμ in a 6 mm. light path.

The results of duplicate analyses on the 129 test specimens are shown (table 1).

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In Vitro Studies on the Effect of Some Antimalarial Drugs on Fasciola Worms in Buffaloes, Cattle, and Sheep

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FASCIOLIASIS, a world-wide animal health problem, causes hepatic and intestinal disorders, as well as losses, in food-producing animals.

The early tendency of Fasciola is to localize embryonically in the parenchymatous hepatic cells. Since this is the same selective habitat of the pre-erythrocytic cryptozoites of plasmodia,[6] the chemotherapeutic value of antimalarial drugs for control of fascioliasis is of interest. Certain antimalarial drugs have been found to have an anthelmintic effect on other intestinal parasites;[1-4,7] *in vitro* studies of the effects of these drugs on Taenia and Ascaris in the dog have been made recently.[5]

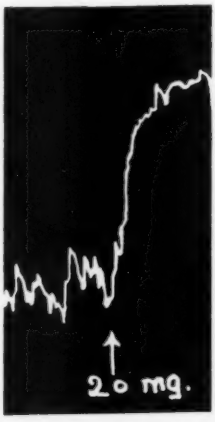

Fig. 1—The stimulation of motility of Fasciola gigantica from buffaloes when 20 mg. of quinacrine was added to the 50-ml. bath (1: 2,500).

It is the purpose of this study to determine the *in vitro* effect of quinacrine hydrochloride (Atabrine),* amodiaquine hydrochloride (Camoquin),† pyrimethamine (Daraprim),** and quinine sulfate on Fasciola flukes collected from buffaloes, cattle, and sheep.

MATERIALS AND METHODS

Fasciola gigantica flukes were collected directly from livers of buffaloes, cattle, and sheep freshly slaughtered at an abattoir. Whole worms were suspended in oxygenated Tyrode's solution at a temperature of about 37 C., using a glass jar bath with a 50-ml. inner vessel. Their movements were recorded on a kymograph.

Quinacrine hydrochloride, amodiaquine hydrochloride, and pyrimethamine tablets (0.1 Gm., 0.2 Gm., and 25 mg., respectively), and quinine sulfate powder B.P. were used to test the response of Fasciola flukes to antimalarial substances. Solutions of these drugs were prepared in Tyrode's solution; insoluble constituents were removed by filtration. Different amounts of these solutions were added to the baths containing the parasites and their effects were recorded (table 1; fig. 1–5).

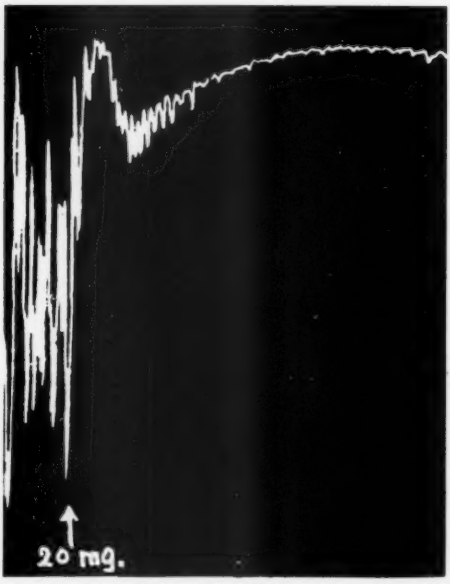

Fig. 2—The stimulation of motility of Fasciola gigantica from cattle when 20 mg. of amodiaquine hydrochloride was added to the 50-ml. bath (1: 2,500).

From the Departments of Pharmacology and Parasitology, Faculty of Veterinary Medicine, Cairo University, Cairo, Egypt.

* Atabrine hydrochloride is produced by Winthrop Laboratories, Inc., New York, N.Y.

† Camoquin hydrochloride is produced by Parke, Davis & Co., Detroit, Mich.

** Daraprim is produced by Burroughs Wellcome & Co., Tuckahoe, N.Y.

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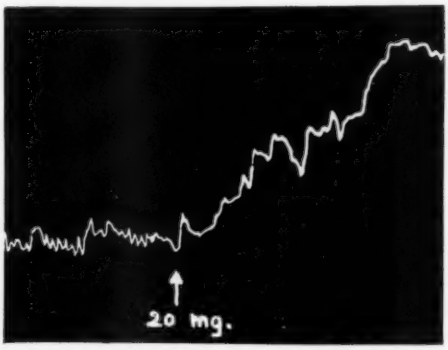

Fig. 3—The stimulation of motility of Fasciola gigantica of sheep when 20 mg. of pyrimethamine was added to the 50-ml. bath (1:2,500).

RESULTS

Quinacrine hydrochloride, amodiaquine hydrochloride, and pyrimethamine produced consistent *in vitro* stimulation of the motility of Fasciola worms.

Quinine sulfate stimulated parasite movement when used in small doses of 0.5 mg. or more; stimulation followed inhibition when quinine was added to the bath in large (5.0 mg. or more) doses. Quinacrine hydrochloride had no effect on Fasciola in amounts up to 0.2 mg. per 50-ml. bath, but increased amounts consistently stimulated motility. When amodiaquine hydrochloride was used, motility was stimulated with doses of 2.0 mg. or more. Pyrimethamine produced *in vitro* stimulation only with 5 mg. or more.

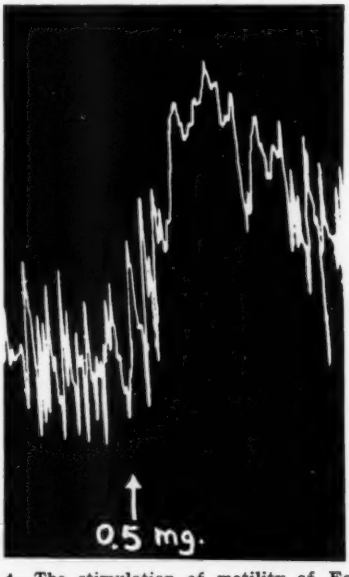

Fig. 4—The stimulation of motility of Fascolia gigantica of buffaloes when 0.5 mg. of quinine sulfate was added to the 50-ml. bath (1:100,000).

DISCUSSION

It is evident from the results that all the tissue antimalarial drugs tested stimulated the *in vitro* movements of Fasciola flukes taken from buffaloes, cattle, and sheep. This finding indicates the use of such drugs as anthelmintics against fascioliasis in these animals in order to excite the motility of the worms. These drugs do not kill the parasites but apparently stimulate their musculature. The increased movement may detach their hold on the bile ducts and gallbladder so that they can be easily eliminated. Therefore, it is suggested that a saline cathartic which is known to produce reflex contractions of the gallbladder be given subsequently to aid the expulsion of the parasites with the bile to the intestine.

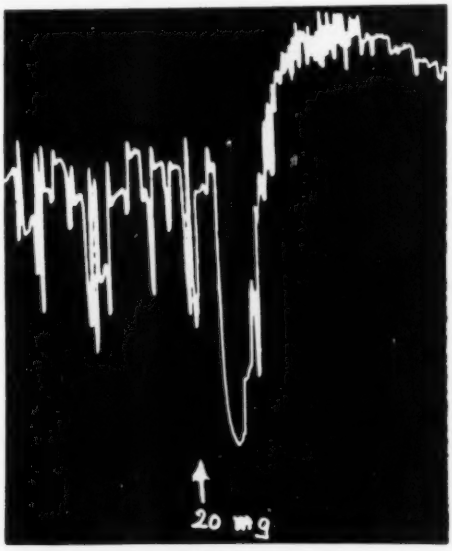

Fig. 5—The stimulation, preceded by inhibition, of the motility of Fasciola gigantica of cattle when 20 mg. of quinine sulfate was added to the 50-ml. bath (1:2,500).

TABLE 1—Comparative Effects of Some Antimalarial Drugs on Fasciola gigantica

Dose (mg.) added to 50-ml. bath	Quinine	Quinacrine	Amodiaquine	Pyrimethamine
0.1	−	−	−	−
0.2	−	−	−	−
0.5	+	+	−	−
1.0	+	+	−	−
2.0	±	+	+	−
5.0	±	+	+	+
10.0	±	+	+	+
20.0	±	+	+	+
50.0	±	+	+	+
100.0	±	+	+	+

+ = stimulation; − = no stimulation; ± = inhibition followed by stimulation.

SUMMARY

Quinacrine hydrochloride (Atabrine), amodiaquine hydrochloride (Camoquin), and pyrimethamine (Daraprim) produced consistent *in vitro* stimulation of the motor activity of *Fasciola gigantica* taken from buffaloes, cattle, and sheep. Quinine sulfate, however, produced stimulation of parasitic movement when used in small doses, and inhibition followed by stimulation when applied in larger amounts.

These antimalarial drugs do not seem to have a lethal effect on the flukes but apparently irritate them, producing excessive movements which may detach their hold on the bile ducts.

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Observations on the Colonial Morphology of Leptospires

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THE FIRST successful growth of leptospires as isolated colonies on a solid medium was reported in a brief note by Cox and Larson.[2] Leptospires were repeatedly grown as discrete colonies on a 1 per cent agar medium containing serum and tryptose-phosphate. Two main types of colonies were observed: one was small in diameter and somewhat opaque, called type 1; the other was of greater diameter, quite translucent and veil-like in appearance, called type 2. Both types of colonies reportedly grew on the surface of the agar.

The present study was undertaken to evaluate the procedure devised by Cox and Larson for the cultivation of leptospiral colonies and to provide basic, descriptive material on the colonial morphology of leptospires. Factors influencing this growth on solid mediums were also investigated.

MATERIALS AND METHODS

A solid medium, modified from that described by Cox and Larson,[2] was used throughout this study. Cycloheximide* was added to suppress the fungal contaminants which threatened to overgrow plates during their long incubation periods, and sheep serum and hemoglobin were substituted for the rabbit serum and sheep hemoglobin used by the previous workers.

Each liter of medium contained 2.0 Gm. of tryptose-phosphate broth (Difco) and 10.0 Gm. of agar (Difco), which were dissolved in 900 ml. of distilled water. After sterilization in an autoclave, the medium was allowed to cool to 50 C. Sterile, Seitz-filtered sheep serum (100 ml.), containing 1 per cent of hemoglobin and 0.1 Gm. of crystalline cycloheximide,* in 10 ml. of sterile distilled water, was added aseptically. The completed medium was then placed in a 56 C. water bath for 30 minutes to inactivate the serum and was poured into Petri dishes. The pH of the medium consistently checked at pH 7.2 to 7.4 and did not require adjustment.

Fifteen strains of leptospires, representing 13 serotypes, were used in this study: *Leptospira biflexa; L. icterohaemorrhagiae,* strain Wijnberg; *L. canicola,* strain R.; *L. bataviae,* strain SvT; *L. grippotyphosa,* strain Moscow V; *L. sejroe,* strain Maller; *L. alexi,* strain Zurite; *L. andaman; L. semerang; L. borincana; L. djasiman; L. javanica;* and *L. pomona,* strains S-91, LC34, and King B.

Some culture mediums were routinely inoculated by spreading them with 0.1-ml. volumes of broth cultures diluted to at least 1:10,000. Others were streaked in the usual manner for isolation with undiluted broth cultures or directly from previously grown leptospiral colonies. Incubation was at 29 C.

A 1 per cent aqueous solution of dimethyl-p-phenylenediamine oxalate, the oxidase reagent, was used to stain transparent colonies in order to increase their visibility and for study of their macroscopic morphology.[4]

In order to observe the microscopic arrangement of the organisms within the colonies, leptospiral colonies were removed from plates in agar plugs, fixed in 10 per cent formalin, and embedded in paraffin. Microtomed sections of these colonies were stained by the Warthin-Starry silver impregnation technique.[6]

In order to acquire some information on colonial respiration, catalase activity was investigated by flooding plates containing colonies with 1 per cent hydrogen peroxide. Plates of medium streaked with *Bacillus subtilis* served as controls.

RESULTS

With two exceptions, each of the 15 leptospiral strains had a predominant colony type with sufficient regularity to be considered characteristic.

Two morphologic forms consistently appeared on plates inoculated with *L. semerang* and *L. biflexa* (fig. 1). The small button-like colonies had entire edges and were quite dense, especially in their centers. The larger, ringworm-like colonies had homogeneous, translucent centers encircled by very narrow rings of almost opaque growth which expanded as the colonies increased in size. These two colonies are similar to types 1 and 2, reported by Cox and Larson [2] as being the most typical forms observed in their laboratory. Zones of growth inhibition between closely adjacent colonies were frequently observed among these colonies particularly, but also with all others. Colonies of *L. biflexa* and *L. semerang* usually became visible on the fourth day of incubation.

From the Department of Microbiology, School of Medicine, University of Missouri, Columbia.

Presented in part at the 59th General Meeting of the Society of American Bacteriologists, May, 1959, at St. Louis, Mo.

* Actidione, produced by The Upjohn Co., Kalamazoo, Mich.

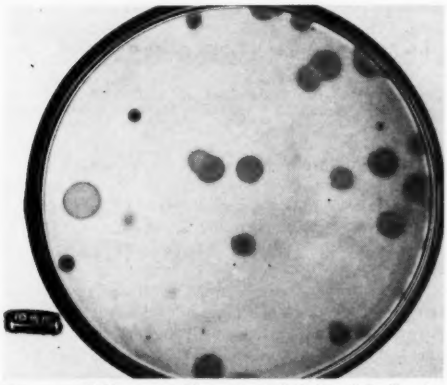

Fig. 1—Culture medium inoculated with Leptospira semerang showing button-like and ringworm-like colonies; stained with oxidase reagent.

Asteroid colonies (fig. 2) were produced by *L. canicola* and *L. bataviae*. These colonies usually became visible 12 to 15 days after the plates were inoculated and developed as multiple, tiny, white granules surrounded by halos of more diffuse growth which gave them a star-like appearance. Even after prolonged incubation, these colonies rarely exceeded a diameter of 3 mm.

Rough, opaque colonies (fig. 3) were characteristically formed by the S-91 strain of *L. pomona* and, occasionally, by *L. bataviae*. These rough colonies, which looked like small, ragged bits of cotton, also required an incubation period of about two weeks before they could be seen and rarely reached a diameter of more than 3 mm.

Rough, translucent colonies (fig. 4) were typical of *L. andaman, L. sejroe, L. grippotyphosa, L. borincana, L. alexi, L. javanica,* and *L. pomona* strains LC34 and King B. These colonies were quite translucent with indistinct and somewhat irregular edges. Examination of their structure was greatly facilitated by flooding them with the oxidase reagent. Although *L. andaman* became visible on the fourth or fifth day, the other serotypes having this characteristic morphology required from nine to 13 days' incubation before growth appeared.

Swarming, Proteus-like growth (fig. 5) was most often produced by *L. icterohaemorrhagiae* and *L. djasiman*. This growth was transparent and nearly invisible without the aid of the oxidase reagent. Only the rounded, well-defined margins of these colonies could be seen unstained. By using the oxidase reagent, growth of the Proteus-like type could first be detected after ten to 12 days of incubation.

It should be emphasized that, although the colony types described were characteristic of the serotypes listed with them, variations in morphology were common. The 15 leptospiral strains are matched with the six colonial types shown in this study (table 1).

Innumerable examinations of all types of leptospiral colonies consistently revealed growth which was entirely subsurface. Agar plugs were dissected from cultures with a scalpel and placed on their sides where they were observed with the 10 x magnification of a stereoscopic microscope. A vertical cross-section of a plug of agar contain-

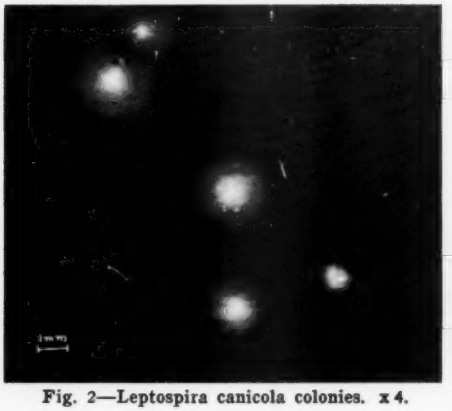

Fig. 2—Leptospira canicola colonies. x 4.

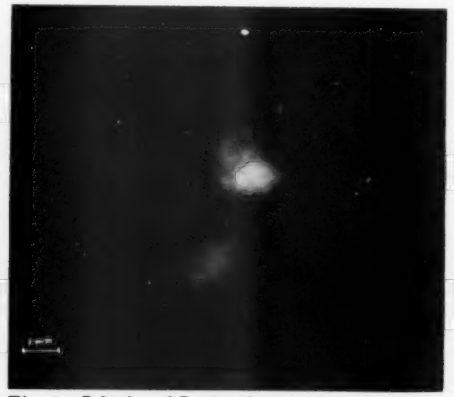

Fig. 3—Colonies of Leptospira pomona, strain S-91. x 5.

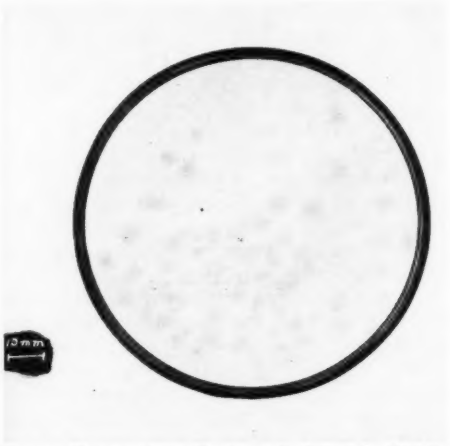

Fig. 4—Leptospira andaman colonies stained with oxidase reagent.

Fig. 5—Culture medium inoculated with Leptospira icterohaemorrhagiae showing Proteus-like colonies; stained with oxidase reagent.

ing a small, button-like colony is shown (fig. 6). The colony, which measured 3.0 mm. across the top and 1.3 mm. in depth, was roughly the shape of a cup and was situated immediately beneath the surface of the agar. The other five colony types also appeared to be somewhat cup-shaped in cross-sectional view and were definitely subsurface in all cases.

The microscopic appearance of the edge of a silver-stained, ringworm-like colony is shown (fig. 7). A dense band of leptospires may be seen bordering an area containing relatively fewer leptospires cells. The band corresponds to the ring of growth which stains darkly when treated with the oxidase reagent, and the area with fewer organisms corresponds to the large central portion of "ringworm"-type colonies which stains lightly or not at all with the oxidase reagent.

No catalase activity was demonstrated on plates of leptospiral growth flooded with 1 per cent hydrogen peroxide. Control cultures streaked with catalase-positive *B. subtilis* produced gas bubbles in profusion when treated similarly.

DISCUSSION

The value of the oxidase reagent as an indicator for three (fig. 1, 4, 5) of the six morphologic colony types cannot be over-

TABLE 1—Colony Types Exhibited by Fifteen Cultures of Leptospires with Their Relative Frequency of Occurrence

Leptospira	Colony types exhibited					
	Buttonlike	Ringworm-like	Asteroid	Rough opaque	Rough translucent	Proteus-like
bifleza	+++	+++	0	0	0	0
semerang	+++	+++	0	0	0	0
canicola	0	0	+++	0	0	0
bataviae	0	0	+++	++	+	0
pomona, S-91	0	0	0	+++	+	0
andaman	+	0	0	0	+++	+
grippotyphosa	++	+	0	0	+++	0
javanica	+	0	0	0	+++	0
borincana	+	0	0	0	+++	0
nejroe	+	0	0	0	+++	0
alexi	0	0	0	0	+++	+
pomona, LC34	0	0	0	0	+++	+
pomona, King B	+	0	0	0	+++	++
icterohaemorrhagiae	++	0	0	0	++	+++
djasiman	+	0	0	0	++	+++

+++ = Characteristic; ++ = occasionally observed; + = rarely observed; 0 = not observed.

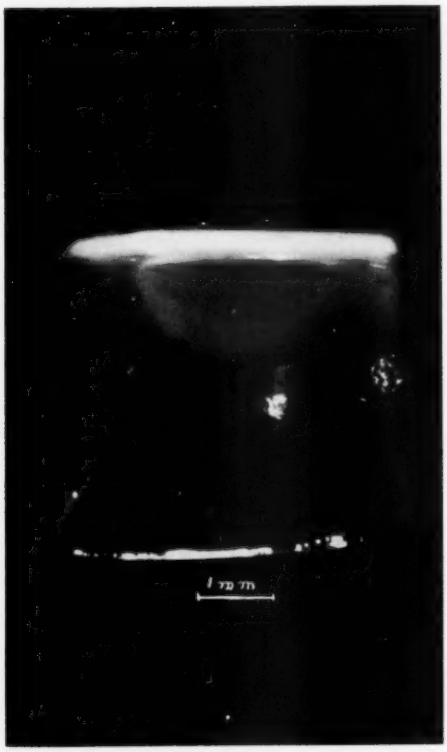

Fig. 6—Vertical cross-section of an agar plug containing a colony of Leptospira biflexa. x 11.

emphasized. With the aid of this reagent, colonies were frequently detected in apparently sterile mediums.

Two of the six colony types observed appear to be facsimiles of the forms illustrated by Cox and Larson.[2] The remaining four types are previously unreported, although the "rough variants" mentioned (but not illustrated) by those authors may have resembled either the rough, opaque (fig. 3) or the rough, translucent (fig. 4) colonies observed in this laboratory.

It would appear that many factors serve to complicate the growth and influence the morphology of leptospiral colonies as observed in this study. Leptospires are in perpetual, rapid motion in a liquid medium and may be seen to penetrate 1 per cent agar with ease. Differences in their motility may partly account for the variety of structure assumed by these colonies. Colonies of Proteus and other actively motile organisms are regulated in size and density by the concentration of agar in the medium.[5] Colonies are more compact in a firm medium and more diffuse in a softer medium. The long incubation periods required by the slow-growing leptospires result in a gradual drying and hardening of the agar which arrests the motility of the spirochetes to some degree.

Zones of growth inhibition between closely adjacent colonies were described by

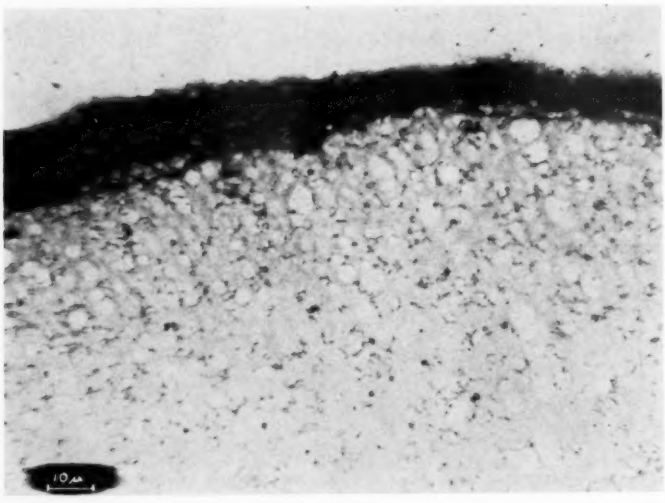

Fig. 7—Horizontal cross-section of a Leptospira semerang colony of the ringworm type. Silver stain; x 646.

Cox and Larson [2] and have also been observed in the present study. This inhibitory effect may be due to the diffusion of a true autolysin into the medium. In line with this, the oxidase-negative central area of a ringworm-like colony (fig. 7) may be the result of autolysis of the older cells at a rapid rate, leaving only a ring of multiplying cells of the periphery. The production of autolytic enzymes under certain conditions may likewise explain the sudden loss of broth cultures and the spontaneous lysis of agglutinated leptospires. Furthermore, leptospires are quickly destroyed in the presence of bile salts.[1]

The evidence that leptospires possess oxidase(s) but apparently lack catalase and form subsurface colonies indicates that these organisms have a somewhat unusual respiratory mechanism. Although Lawrence [7] was able to grow two strains of leptospires in atmospheres with oxygen contents of 0.5 to 90.0 per cent, the O-R potential at which optimal growth occurred was found by both Lawrence [7] and Czekalowski [3] to coincide with that of strict aerobes. Thus, the leptospires have characteristics of obligate aerobes and also of microaerophiles.

It is believed, therefore, that the factors of motility, agar concentration, autolysins, and oxygen-tension influence the formation of leptospiral colonies. In addition, other variables, such as pH and surface tension, may exert an effect on these colonies.

Summary

In a study made of the colonial morphology of leptospires on a solid medium, the 15 strains examined were regularly characterized by six morphologic types of colonies.

The oxidase reagent, dimethyl-p-phenylenediamine oxalate, selectively stained all leptospiral growth and greatly facilitated the examination of translucent colony types. Although the colonies showed wide variation in size, shape, density, and growth rate, they were similar in that they grew entirely subsurface and were more or less cup-shaped in cross-section.

Some of the factors influencing colonial variation, such as motility, autolysis and oxygen-tension, were discussed.

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Scientific Notes

Eradication of the Tropical Rat Mite Ornithonyssus bacoti (Hirst, 1913) from a Colony of Mice

JAMES M. HARRIS, D.V.M., and JACK J. STOCKTON, D.V.M.

THE MAINTENANCE of animal colonies free of ectoparasites requires constant vigilance and strict sanitary and precautionary measures to lesson the probability of introducing parasitic arthropods. Despite such measures, infestations occasionally appear and drastic procedures may be necessary to eradicate the intruder.

The tropical rat mite, Ornithonyssus bacoti, is an especially troublesome pest of laboratory animals, particularly rats, mice, and hamsters. The general health of animals with heavy infestations is markedly affected; Olson and Dahms [1] have reported death due to chronic blood loss. Strong [2] considered this pest to be a serious problem and experienced difficulty in controlling it.

Although the colony involved in the present experiment was small, it was felt that the methods used for eradication of mites could easily be adapted to a larger scale of operation.

COLONY ORGANIZATION

The colony in which mice became infested was established on September 1, 1957. Three animal rooms comprised the installation. In addition, a cleaning and storage room was shared with colonies of rabbits, guinea pigs, and hamsters. Each room had a drain in the center of the concrete floor; the lower halves of the walls were tiled and the upper halves and ceilings were painted plaster. All rooms entered into a common hallway; doors to each room were metal with a concrete sill four inches high under each.

As of September 7, the main colony room contained 600 breeding mice housed in 51 metal cages. Wood shavings were used as litter and the cages were kept on metal racks. There were also three wooden desks in this room for supplies and records. The second room was used to house pregnant mice and suckling mice that were being raised for the breeding colony. The third room held suckling mice being used for experimental purposes. In each room, food and wood shavings were kept in metal containers. A refuse can was also kept in each room.

From Michigan State University, East Lansing, where Dr. Harris was a senior student while this work was being done. His present address is 6947 Pasa Robles Drive, Oakland, Calif. Dr. Stockton is associate professor, Department of Microbiology, Michigan State University.

From September 1 to December 15, mortality from all causes was 14 per cent among suckling mice being raised for the breeding colony, as determined at weaning time. Sometime during the latter part of September or the early part of October, O. bacoti was introduced into the colony. The origin of the infestation is unknown. During November and early December, the increase of mites was so severe that personnel working with the colony were constantly bothered by mite bites when handling mice or cages or when merely present in the room for any length of time. By mid-December the mites had become so numerous that they were easily observable on the walls, racks, and equipment, and some of the workers were showing sensitivity to mite bites. Both adult and suckling mice were anemic from the feeding activities of the mites and the percentage of mortality among suckling mice and young weanlings had risen sharply.

It was evident that drastic measures were necessary to eradicate the mites; the problem was how to exterminate them with the least possible adverse effect on the mice. Literature offered little in the way of specific recommendations, so several schemes were devised for comparative studies.

PROCEDURE and RESULTS

Rooms and Equipment.—The rooms were emptied and all cages were boiled for 30 minutes in live steam-fed tanks. Water jars and other boilable equipment were similarly handled. The wooden desks were removed permanently and the floors, ceilings, walls, and racks were washed with hot water and a brush. The rooms, racks, and cages were sprayed with a 2.5 per cent chlordan solution. This spray was used in all air vents in the rooms and, to create a safety zone, the hallway was also washed and sprayed. This procedure was found completely effective in removing the mites from the rooms and equipment. The cages were washed thoroughly in water on January 7 to remove any residual chlordan.

Animals.—Mice were given various treatments as follows:

(1) A group of suckling mice, 7 to 14 days old, and their dams were dusted with powder containing 1.6 per cent rotenone and 2.6 per

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cent of other cube root extracts. The powder was also mixed into the wood shavings. Nervous symptoms were noticed in the young mice in about ten minutes and death followed shortly.

(2) The same treatment given to 75 adult breeding mice resulted in severe conjunctivitis with pasting of the lids in over 50 per cent of the animals. These mice recovered in a few days, and no deaths were observed.

(3) A group of adult mice were dusted with 1 per cent lindane powder and placed in cages, the bottoms of which had been dusted with 1.6 per cent rotenone and covered with fresh wood shavings. No adverse effects were noticed in these mice.

(4) Powder containing 1 per cent lindane was dusted on 7-day-old suckling mice and their dams. In some litters, central nervous system disorders developed in about ten minutes in the suckling mice and death followed. Mice in other litters showed no ill effects from this treatment.

(5) Powder containing 1 per cent lindane was dusted on the dams and also placed on the bottoms of the cages and covered with wood shavings. Suckling mice and their dams were then placed in these cages. A few young mice developed central nervous system disorders and subsequently died.

(6) Powder containing 1 per cent lindane was placed in the bottoms of cages and covered with wood shavings. Suckling mice and their dams were then placed in these cages. Although a few of the suckling mice died (3 of 41) before weaning, none showed any evidence of insecticide poisoning.

The results of these trials are shown (table 1). All of the schemes tried resulted in complete removal of mites from the mice.

DISCUSSION

Olson and Dahms [1] suggested the use of 10 per cent DDT powder applied to rats and hamsters for the control of mites. This chemical is harmful to mice, and Strong [2] recommended the use of a mixture of equal parts of talcum and powdered derris root with a small quantity of the detergent 2:6 dimethylnaphthalene added. Weinman [3] indicated that DMC (dextrose, magnesium, and calcium) and aramite are useful for control. However, the literature reports were meager and, since little or nothing was found concerning colony management, we decided to try a number of approaches using insecticides readily available to us.

As previously indicated, the mortality in suckling mice from September 1 to the start of the experiment on December 15 was 14 per cent, as determined at weaning. These deaths were due to undetermined causes and included the so-called "normal death loss" in such a colony. Severe acariasis surely was a factor in those animals dying after December 1, when the infestation became so grossly evident.

The percentage of mortality as determined at weaning rose to 100 per cent and was 64.0 per cent on January 7. This was due to the toxicity of the substances being tried in the eradication and not to the mites, since the treatments used were completely effective in removing the mites. By January 30 the mortality had dropped to a low of 7.3 per cent, and it has been maintained at about this level since. The slight drop in mortality (10.2 to 7.3%) that appeared in weanling mice after washing the chlordan-

TABLE 1—Comparison of Different Treatment Schemes in Suckling Mice Infested with Ornithonyssus bacoti

Date of birth	Date weaned	No. of litters	Treatment scheme*	No. dead / No. born	Mortality at weaning (%)
12/1 to 12/15	.....	10	Dust of 1.6% rotenone and 2.6% other cube roots on suckling mice and dams. Dust mixed with wood shavings. In chlordan-sprayed cages.	103/103	100.0
12/15	1/7	12	1% lindane on dams and on suckling mice. In chlordan-sprayed cages.	64/100	64.0
12/17 to 12/21	1/14	15	1% lindane on dams and on suckling mice. In chlordan-sprayed cages until 1/7.	74/125	59.2
12/22 to 12/29	1/22	12	1% lindane on dams and on bottoms of cages under wood shavings. In chlordan-sprayed cages until 1/7.	10/98	10.2
1/4 to 1/7	1/30	5	1% lindane on botttoms of cages under wood shavings. Chlordan-sprayed cages washed before mice placed in them.	3/41	7.3

* Chlordan-sprayed cages were washed thoroughly in water on January 7 to remove any residual chlordan.

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sprayed cages on January 7 might indicate some slight toxicity due to residual chlordan.

It is felt that the use of either of the insecticides (rotenone and lindane) tried in these experiments directly on suckling mice is contraindicated. Of 36 mice (weaned Jan. 7) and their dams, both dusted with 1 per cent lindane, 19 females and 3 males were dead by January 15. Death was thought to be due to the cumulative action of the insecticide. Substantiating this opinion was the observation that in those groups in which suckling mice were not dusted directly, but lindane was used on the dams or under the wood shavings, or both, the majority of deaths in the young mice occurred in the third week of life. Adult mice tolerated dusting with 1 per cent lindane well, and this insecticide is advocated for use in the breeding stock and experimental stock. For use in cages containing young mice and their dams, the use of small amounts of 1 per cent lindane powder under the wood shavings is recommended.

When a colony is clean, every attempt should be made to keep it that way. All of the known rules of sanitation should be strictly adhered to, and any additions to the colony should be dusted on arrival and held in quarantine a few days before being placed in the colony. Occasion use of 1 per cent lindane powder under the wood shavings will, in addition to helping control acarids, aid in eliminating other ectoparasites which may become a nuisance.

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Further Notes on the Exsheathing Mechanisms of Third-Stage Larvae of Haemonchus contortus

ALAN TAYLOR and J. H. WHITLOCK, D.V.M., M.Sc.

Ithaca, New York.

Earlier we reported that we had been able to induce exsheathment of third-stage Haemonchus contortous larvae by subjecting them to anaerobic cultures of Aerobacter aerogenes grown in Simmon's citrate mediums.* As a result, we indicated that some natural conditions giving rise to exsheathment are likely to be rather simple and nonspecific.

We now report that carbon dioxide, salt, and water at 37 C. seem to form a simple, effective, ensheathment system. High rates of exsheathment can be obtained in 0.5 per cent solutions of NaCl, into which carbon dioxide has been bubbled for five minutes. Aliquot amounts of KCl, NaBr, and $NaHCO_3$ seem to be equally effective, but saturated solutions of CO_2 in distilled water are ineffective.

This investigation was supported in part by a research grant, PHS 739, from the National Institutes of Health, Public Health Service.

* Whitlock, J. H., Taylor, A., and Conway, D.: A Note on Exsheathing Mechanisms of Third-Stage Larvae of Haemonchus contortus. Cornell Vet., 49, (July, 1959): 421-422.

Meetings • • •

• • • Symposium on Continuous Culture of Micro-Organisms. London, March 31-April 1, 1960. R. Elsworth, Ministry of Supply, Microbiological Research Establishment, Porton, Salisbury, Wilts, England, secretary.

• • • American Society for Experimental Pathology. Chicago, April 11-15, 1960. Dr. J. F. A. McManus, University of Alabama Medical Center, Birmingham, executive secretary.

• • • International Academy of Pathology. Annual meeting, April 24-26, 1960, Memphis, Tenn.; scientific meeting, June 20-24, 1960, London. Dr. F. K. Mostofi, Armed Forces Institute of Pathology, Washington 25, D.C., secretary.

• • • International Meeting on Veterinary Education. London, April 25-29, 1960. Food and Agriculture Organization of the United Nations, Viale delle Terme di Caracalla, Rome, Italy.

• • • American Association of Pathologists and Bacteriologists. Memphis, Tenn., April 28–30, 1960. Dr. Russell L. Holman, Department of Pathology, Louisiana State University School of Medicine, New Orleans, La., secretary.

• • • Society of American Bacteriologists. Philadelphia, Pa., May 1–5, 1960. Dr. E. M. Foster, 311 Bacteriology, University of Wisconsin, Madison 6, secretary.

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• • • American Federation for Clinical Research. Atlantic City, N.J., May 2, 1960. Mr. James E. Bryan, 250 W. 57th St., New York 19, N.Y., executive secretary.

• • • Pfizer Annual Research Conference. Terre Haute, Ind., May 6, 1960. H. G. Luther, Agriculture Research and Development Center, Chas. W. Pfizer & Co. Inc., Terre Haute, Ind.

• • • Rare Earths in Biochemical and Medical Research Conference. Iowa State University, Ames, May 11–13, 1960. Dr. J. G. Graca, College of Veterinary Medicine, Iowa State University, Ames, program chairman.

• • • International Association of Veterinary Food Hygienists, Second Symposium. Basel, Switzerland, May 15–21, 1960. Dr. A. Clarenburg, 1 Sterrenbos, Utrecht, Netherlands, president.

• • • International Congress of Clinical Pathology. Madrid, Spain, June 13–17, 1960. Dr. J. Aparicio Garrido, Sandoval 7, Madrid, Spain, secretary-general.

Training Program for Steroid Biochemistry

To alleviate the shortage of qualified trained personnel in steroid research, a training program will be sponsored by the U.S. Public Health Service through the National Cancer Institute of the National Institutes of Health. The courses will stress theoretical and practical aspects as well as specialized laboratory techniques in steroid research.

Classes for the 1960-1961 session will begin Oct. 1, 1960. They will be held at the Worcester Foundation for Experimental Biology, Shrewsbury, Mass.; the Department of Chemistry, Clark University, Worcester, Mass.; and the Department of Biochemistry, College of Medicine, University of Utah, Salt Lake City.

Post-doctoral candidates (M.D. or Ph.D.) selected for admission at either Worcester or Salt Lake City will receive stipends of $5,500 for a one-year period. Pre-doctoral candidates (B.S., M.S., or equivalent) selected for admission (only at Worcester) will receive stipends of $1,800 for a six-month period.

The final date for completion of application is June 1, 1960. Inquiries and requests for applications should be directed to Dr. Kristen Eik-Nes, Department of Biochemistry, College of Medicine, University of Utah, Salt Lake City 10, or to Dr. William R. Nes, Department of Chemistry, Clark University, 950 Main Street, Worcester, Mass.

Correction—Chlorpromazine Premedication in Mice

We regret the error in the title of the paper "Chlorpromazine Premedication with Pentobarbital Anesthesia in a Mouse," by William C. Dolowy, Paola Mombelloni, and A. L. Hesse, published in the Scientific Notes section (p. 156) of the January, 1960, issue of the American Journal of Veterinary Research. The title should read "Chlorpromazine Premedication with Pentobarbital Anesthesia in the Mouse."

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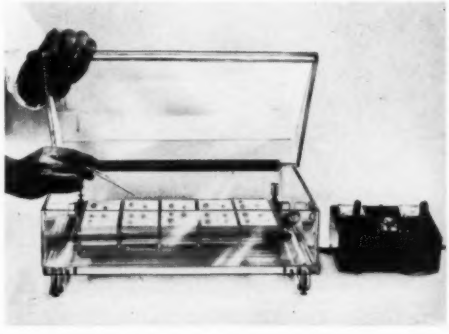

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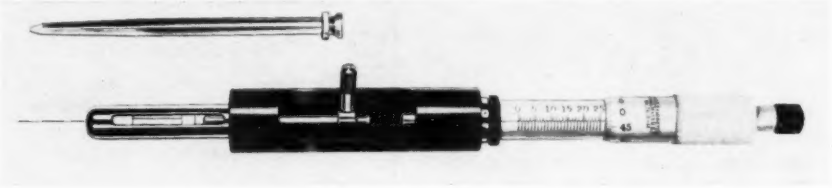

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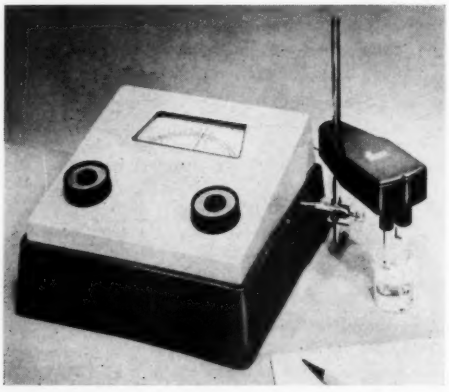

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*Journal A.V.M.A. 134:155-161 (Febr. 15) 1959

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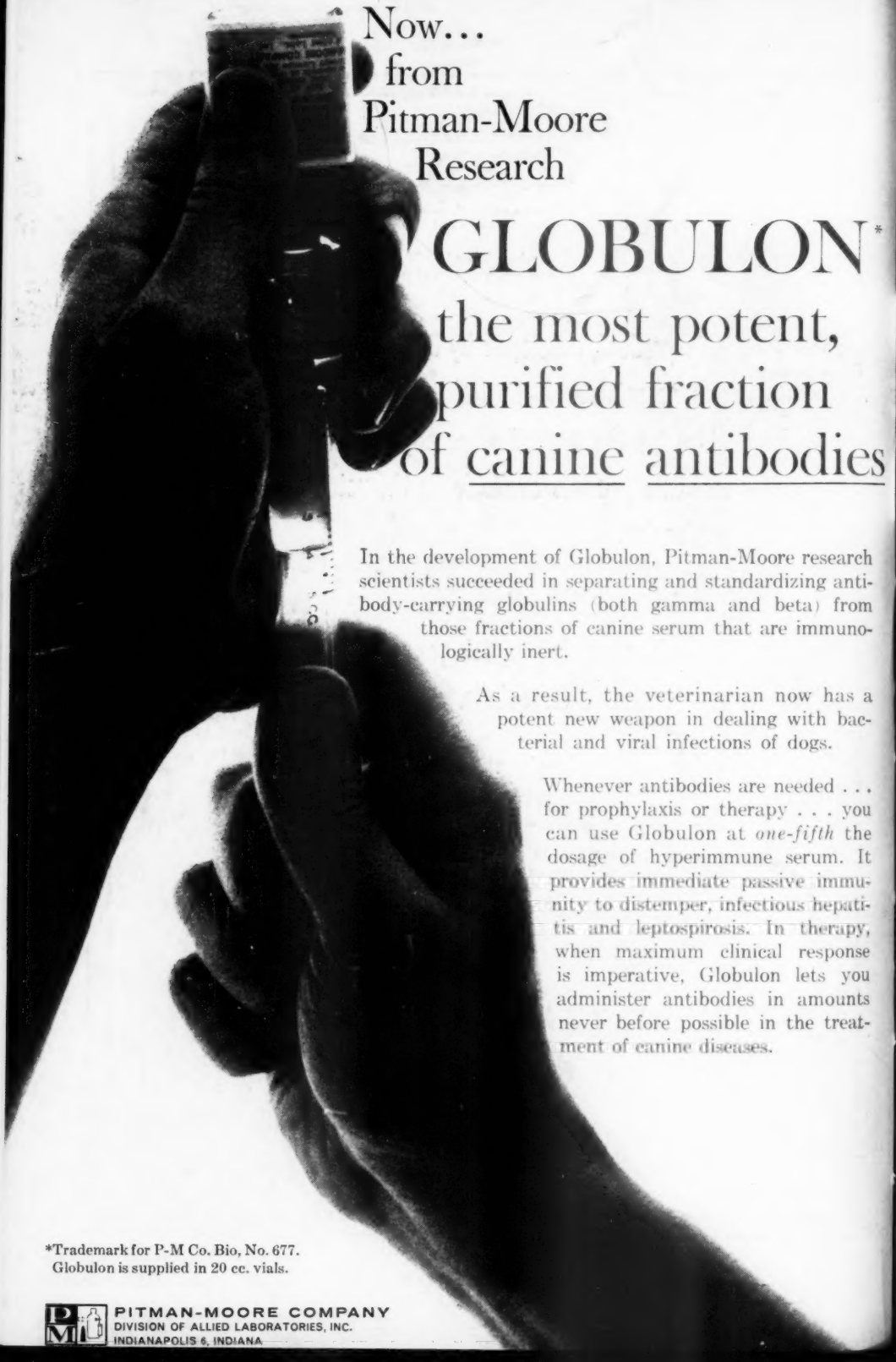

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